REMARKS

Claims 2-5, 8-10, and 34-48 stand rejected under 35 U.S.C. §103(a) as being obvious over U.S. Patent Application Publication 20030100752 published May 29, 2003 in the name of Robinson (hereinafter "Robinson"), in view of Drummond *Control of Heme Metabolism by Synthetic Metalloporphyrins*, Annals of New York Academy of Sciences, 1987, 514, 87-95 (hereinafter "Drummond") and Bettelheim *et al.*, *General, Organic and Biochemistry*, 1998, page 596 (hereinafter *Bettelheim*).

Claims 2-5, 8-10, and 34-48 are hereby canceled, new claims 49 – 70 are hereby added, and the rejections are hereby traversed as if having been applied to the *new* claims.

I. The New Claims are not obvious over Robinson in view of Drummond and Bettelheim.

As regards the rejection of the claims for obviousness over Robinson in view of Drummond and Bettelheim the Examiner argues as follows:

> "**Robinson teaches tin porphyrin complexed with amino acid (page 45, claim 1), wherein the substitutions R1-R12 can be H, alkyl or alkenyl and could also be substituents that carry amino acid residues (page 46, left column, middle; page 34, left column, paragraph 200)**. One of the preferred metal ions complexed to the core is Tin (Symbol for Tin is Sn; page 32, paragraph 183). Formulations comprising the porphyrins of Robinson's invention can be optimized to contain between 0.1 and 50 mg (page 33, paragraph 193). **The compounds of Robinson can be applied as aqueous solutions (page 35, paragraph 211). This means that the tin porphyrins of Robinson's invention are water-soluble.** Robinson's compounds can have CO_2R_{13}, wherein R_{13} can be H, i.e., a COOH group (page 45, right column, line 10) as a substituent for R_8 and R_{10} in his compound of formula I. Mesoporphyrins have a $-(CH_2)_2COOH$ group for R_8 and R_{10}.
>
> **The only difference between the instant compounds claimed and that of Robinson is the alkylene group**. One of ordinary skill in the art would expect tin mesoporphyrins having the $-(CH_2)_2COOH$ group for R_8 and R_{10} also to be water soluble when complex with amino acids. According to Robinson the water-soluble tin mesoporphyrin analogs complexed with amino acid are useful for treating psoriasis (page 35, paragraph 211).

Drummond, drawn to metalloporphyrins, teaches control of heme metabolism using tin-protoporphyrins (page 87, introduction, last paragraph). Tin protoporphyrin was by far the most potent (page 88, Results and Discussion; page 89). **Tin protoporphyrin is structurally similar to tin-mesoporphyrin except that the protoporphyrin has an ethylene side chain. In the mesoporphyrin, the ethylene side chain is saturated (ethylene side chain if saturated becomes ethyl group). According to Robinson (above), the side chain can be an alkenyl or alkyl. When it is alkenyl, the structure is similar to the protoporphyrin as taught by Drummond, which can be converted to ethyl via hydrogenation**.

According to Drummond his synthetic heme analogs have novel biological properties and may have useful clinical roles (see Introduction) and tin-protoporphyrins have proved to be innocuous in toxicology studies in animals. Long-term treatment of rats with tin-protoporphyrins resulted in decrease in bilirubin levels (page 90, middle paragraph; page 92, first full paragraph).

Bettelheim in general teaches that amino acids exist as zwitterions and are polar. This renders them water soluble. From this and the teaching of Robinson, one of ordinary skill in the art will recognize that complexing the tin-porphyrins of Robinson with amino acids will enhance their solubility and also the solubility of the corresponding mesoporphyrins. Robinson also teaches the use of aqueous solutions of the porphyrins for administration.

Based on the teachings of the prior art above, it would have been obvious to one of ordinary skill in the art at the time the invention was made to make tin mesoporphyrins comprising amino acid residues complexed to the porphyrins and their compositions with a reasonable amount of success since structurally close water soluble analogs complexed with amino acids are seen to be taught in the prior art.

One of ordinary skill in the art would be motivated to make tin mesoporphyrins as instantly claimed because the structurally analogous water soluble tin porphyrins, as taught by Robinson and Drummond, are not toxic and have useful therapeutic properties and complexing amino acids to the porphyrins would enhance their aqueous solubility and hence their bioavailability.

Even though Drummond teaches protoporphyrins, Robinson's teaching embraces both proto and mesoporphyrins. Proto- and mesoporphyrins are known in the art and are recognized as interchangeable because of their structural similarity. Hence one of ordinary skill in the art would reasonably expect mesoporphyrins as instantly claimed to have the same or substantially similar beneficial therapeutic effects as taught by Robinson and Drummond. One of ordinary skill in the art would also extend the teachings of the prior art to making tin mesoporphyrins as instantly claimed in order to look for more active compounds.

Response to Applicants Arguments

Applicants have traversed the rejection of record arguing that:

1. The issue presented is whether the art teaches one skilled in the art that the water insoluble mesoporphyrin should be complexed with an amino acid

and that such complexing would impart water solubility to tin mesoporphyrin.

2. **Robinson does not disclose or suggest such compounds should be complexed with an amino acid and/or that such a complex would be water soluble even though the uncomplexed compound is water soluble.**

3. **Drummond does not disclose a tin mesoporphyrin and does not disclose or suggest that such a complex would be water soluble even though the uncomplexed compound is water soluble.**

4. Bettelheim discloses that some amino acids are water soluble. This does not suggest or render obvious applicants invention.

Applicants' arguments are not found to be persuasive.

Even though Drummond teaches protoporphyrins, Robinson's teaching embraces both proto and mesoporphyrins. Proto- and mesoporphyrins are known in the art and are recognized as interchangeable because of their structural similarity. The compounds of Robinson and Drummond have very closely related structural core. One of the substituents in Robinson's porphyrins is amino acids. Robinson teaches that his compounds can be applied as aqueous solutions (page 35, paragraph 211). This means that the tin porphyrins of Robinson's invention including the ones having amino acids as substituents are water-soluble. Since Bettelheim teaches that amino acids are polar and this makes them water soluble it is logical to choose amino acids as substituents from among the substituents taught by Robinson in order to make mesoporphyrins more water soluble. There is suggestion in the prior art as to the preference of amino acids as substitutions and tin as the metal atom in the porphyrin core to make them water soluble. A reasonable expectation of success is also seen based on the teachings of the prior art." (Instant Office Action pages 3-7; Applicants emphasis added).

In response, Applicants first note that Robinson does not teach "tin porphyrin complexed with amino acid." As the above-quoted Office Action concedes, Robinson teaches nothing more than that Robinson's R groups (R_1-R_{12}) may contain "substituents that carry amino acid residues[.]" As the term "residue" clearly denotes, Robinson's R groups may be amino acid *functionalized* through *covalent* bonding of the amino acid to the R group:

"**Residue**

A residue in normal English speech is a product, usually unwanted, left behind by any process. In mathematics: In complex analysis, the residue is a complex number which describes the behavior of line integrals of a meromorphic function around a singularity. In modular arithmetic, the residue of an integer n to base b is the remainder r after the largest multiple mb of b no greater than n has been subtracted from n. (If n < 0, one adds multiples of b just

> sufficient to make the result non-negative.) The residues modulo (to the base) b form a ring. In a locally ringed space (X, OX), the residue of a section f in OX(U) at a point p of X is the reduction of the germ of f at p modulo the unique maximal ideal of the stalk of OX at p. When OX is a sheaf of functions on X, the residue of a section f at a point p is the value f(p). **In general chemistry, a residue is what is left behind by a reaction**. In chemistry a portion of a larger molecule is often called a residue. See residue (chemistry). In petroleum refining the residue comprises the heavier fractions that fail to vaporize. It can be used as fuel oil or cracked to produce lighter fractions. The volume and degree of cracking carried out is determined by the price difference between the light and heavy fractions and the cost of the cracking process. (*See* Babylon English English Dictionary, available at http://www.babylon.com/definition/residue/English, hereinafter "Babylon," attached hereto as **Exhibit A**; Applicants' emphasis added).

As is known in the art, the anionic *ligand* of a *complex* is entirely different from a covalently bonded *residue*:

> "A complex in chemistry and biochemistry is a reversible association of molecules, atoms, or ions through weak **non-covalent chemical bonds**. Simple salts are usually not considered complexes." (*See* Chemistry Daily, available at http://www.chemistrydaily.com/chemistry/Complex_(chemistry), hereinafter "Chemistry Daily," attached hereto as **Exhibit B**; Applicants' emphasis added).

To be sure, none of the Examiner's citations to Robinson in any way discloses an amino acid acting as *anion* with respect to the porphyrin ring's Tin acting as a *cation*, which **is** the relationship contemplated by both the chemistry of *complexes* and the *scope* of the instant claims.

In further response Applicants note that nowhere in this prosecution history have Applicants conceded that either tin protoporphyrin or tin mesoporphyrin is **water-soluble**. It is indeed noted in the instant specification that tin mesoporphyrin is **water-insoluble**:

> "[0005] **The above-referenced methods for the preparation of the stannsoporfin, or tin (IV) mesoporphyrin IX, however, result in a non-water soluble compound**. Non-water soluble compounds are difficult to use as therapeutic agents, absent special delivery modes, such as encapsulation into a tablet or capsule or via use as a powder. Applications of stannsoporfin in therapeutic treatment of conditions affecting neonates, children, and adults have thus been hindered." (*See* ¶[0005] U.S. Patent Application Publication

20040097481; Applicants' emphasis added).

In further response, Applicants note that the new claims are directed to water-soluble tin mesoporphyrin compounds comprising a tin mesoporphyrin *component* complexed with at least one amino acid *component*, wherein the compound is water-soluble, and wherein the tin mesoporphyrin *component* has the specific formula depicted in Figure 1 of the instant specification (and in new claims 49, 52, and 56), or wherein the tin mesoporphyrin *component* is specifically tin (IV) mesoporphyrin IX dichloride (or stannsoporfin)(as recited in claims 60, 63, 66, and 67)—to be clear, the specific formula depicted in Figure 1 of the instant specification **is** tin (IV) mesoporphyrin IX dichloride (or stannsoporfin).[1] Robinson does not disclose or suggest the tin mesoporphyrin *component* of the specific formula depicted in Figure 1 of the instant specification / tin (IV) mesoporphyrin IX dichloride (or stannsoporfin), let alone its being complexed with an amino acid *component* for purposes of imparting water solubility to it.

The Examiner argues that "[t]he **only** difference between the instant compounds claimed and that of Robinson is the alkylene group." Applicants would note that while they agree with the Examiner that the tin mesoporphyrin component of the instantly claimed complexed compound does differ from tin protoporphyrin by a number of alkene groups that is clearly **not** the only difference between the tin mesoporphyrin component of the instantly claimed complexed compound and the open-ended number of compounds disclosed by Robinson. As can be plainly seen from Robinson's formula I (*See* Robinson's claim 1) the "M," in **further** dissimilarity, is bound to all *four* nitrogen in the porphyrin ring. In stark contrast, as can be seen from Figure 1 of the instant specification (and in new claims 49, 52, and 56), the tin ("Sn") in the

[1] Inasmuch as both set of claims (49-56 and 57-70) are offered in the *same* application they present no double patenting issues, notwithstanding the *identities* of scope.

tin mesoporphyrin *component* of the claimed compound is bound to only *two* of the porphyrin ring nitrogen, while being further bound to *two* separate *chlorides*. And, no structure depicted in Robinson, or hypothetically assembled by the Examiner from the open-ended menu that is its specification and claims, teaches or suggests the tin mesoporphyrin *component* of the compound as instantly claimed. Accordingly, regardless of how many trillions of compounds Robinson's varying **R groups** (R_1-R_{12}) might permit of Robinson to teach or suggest, the tin mesoporphyrin *component* of the compound as instantly claimed is *not* to be counted amongst *their* number, since those **R groups** change *nothing* with regard to the disparate *functionalizing* of the **centralized metal atom** or the disparate *stereochemisty* that likely obtains as a result of that disparate functionalizing. And, inasmuch as post-KSR "it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound[,]" Takeda Chemical Industries, Ltd. v. Alphapharm Pty., Ltd., 492 F.3d 1350, 1356-1357 (Fed. Cir. 2007)(*citing* KSR, 127 S.Ct. at 1731), it must *surely* remain necessary to identify some reason that would have led a chemist to modify a known compound (*i.e.*, one of Robinson's trillions) in a particular manner into another known compound [*i.e.*, the (IV) mesoporphyrin IX dichloride (or stannsoporfin) component of the instantly claimed product] to establish prima facie obviousness of a **combination product**, within which the other known compound is but a mere *component*.[2]

This structural difference is also significant as regards the *method* claims since the Examiner sets substantial store for his argument that the artisan would reasonably expect

[2] Nothing in either Drummond or Bettelheim cures Robinson's shortcomings in this regard as neither Drummond nor Bettelheim disclose or suggest "tin mesoporphyrin," and similarly neither discloses or suggests anything with regard to its being *complexed* with an *amino acid* for purposes of imparting *water solubility*.

Drummond's tin **proto**porphryin and the tin **meso**porphyrin component of the instant claims to have "the same or substantially similar beneficial therapeutic effects" by virtue of their "structural similarity." That expectation theory is, therefore, *undermined* when it is considered that in the instant claim the *structural differences* exceed *several* functionalities rather than just "the alkylene group" that the Examiner strives to marginalize. The expectation theory, however, is completely **disproved** when it is further considered that the art actually *demonstrates* that **virtually identical mesoporphyrins** have wholly *disparate* "therapeutic effects" even when "the alkenyl group" is *common* to both, and the mesoporphyrins differ *only* in their **central metal atom**:

> "**Abstract**
>
> The effects of two synthetic heme analogues, zinc mesoporphyrin (ZnMP) and tin mesoporphyrin (SnMP), on in vivo hematopoietic progenitor cell mobilization and in vitro hematopoiesis were examined in rabbit bone marrow. Rabbits received granulocyte colony-stimulating factor (rhG-CSF) for 7 days in order to mobilize increased numbers of erythroid (BFU-E) and myeloid (CFU-GM) progenitors in peripheral blood. Concurrent treatment of rhG-CSF-treated rabbits with ZnMP reduced mobilization of the numbers of BFU-E (76% inhibition, $p < 0.0001$) and CFU-GM (70% inhibition, $p < 0.005$) in peripheral blood. In contrast, SnMP administered at the same concentration had no significant suppressive effect on BFU-E and CFU-GM recruitment. Both metalloporphyrins inhibited bone marrow heme oxygenase activity equally in vivo, thus indicating that both compounds enter bone marrow cells. Direct in vitro addition of ZnMP to normal rabbit bone marrow cultures suppressed BFU-E and CFU-GM growth, whereas SnMP had no such effect. **These results confirm, in an in vivo system, our earlier in vitro studies and demonstrate that, at the concentrations studied, ZnMP, in contrast to SnMP, displays toxicity for hematopoietic growth and progenitor cell production**." (*See* Lutton *et. al.*, *Comparative Pharmacology of Zinc Mesoporphyrin and Tin Mesoporphyrin: Toxic Actions of Zinc Mesoporphyrin on Hematopoiesis and Progenitor Cell Mobilization*, Pharmacology 1999; 58:44-50, hereinafter "Lutton," attached hereto as **Exhibit C**; Applicants' emphasis added).

Additionally, Drummond does not even *mention* "tin **meso**porphyrin" (or any other **meso**porphyrin) such as could commend its interchangeability with Drummond's tin

protoporphyrin, and in the post-KSR world the Examiner asserting the *interchangeability* of components must *still* provide evidence showing that the art *considered* the components to be *interchangeable* for the claimed purpose:

> "Chien states that the water miscible solvent "increase[s] the aqueous solubility of [the] pharmaceutical" (Chien, col. 3, ll. 21-31). Chien does not teach that the water miscible solvent is PVA or PVP, nor does the Examiner cite any evidence that PVA or PVP would be expected to increase the aqueous solubility of enzymes. **There is also no evidence of record that PVA or PVP was considered to be interchangeable with the glycols described in Chien that would given a person of ordinary skill in the art reason to have substituted one for the other**. *See KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, __, 82 USPQ2d 1385, 1395 (2007). **Therefore, we agree with Appellants that the Examiner has not adequately shown that it would have been obvious to include PVA or PVP in the solvent system described in Chien. We therefore reverse the rejection of claim 22 and of claims 23 and 24, which depend from claim 22.**" Ex parte Bott, 2007 WL 2020937 *8 (Bd. Pat. App. & Interf.)(May 21, 2007)(Applicants' emphasis added).

> "**The Examiner did not rely on any evidence demonstrating that a person having ordinary skill in the art would have reasonably expected that MgR_2 and $Mg(OR^1)_2$ are interchangeable for Gray's purpose, which is to synthesize a Ziegler-Natta olefin catalyst component**.

PRINCIPLES OF LAW

While the Supreme Court of the United States has recently rejected a formalistic and rigid application of the teaching, suggestion, or motivation test as an exclusive test in the obviousness inquiry, it nevertheless made clear that an invention "composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." ***KSRInt'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1741 (2007)**. The Supreme Court elucidated on this matter by stating that "it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine elements in the way the claimed new invention does." *Id.*

ANALYSIS

The Examiner appears to realize that neither reference describes the use of $M(OR^1)_2$ as a reactant. To make up for this deficiency, the Examiner speculates as to the structure of the reaction product of sodium ethoxide with diketone described in Luinstra at paragraph 0078. (Ans. 3.) The Examiner then alleges that "[b]oth sodium (Na^+) and magnesium (Mg^{2+}) belong to Groups 1A and 2A

metals respectively and their analogous compounds are expected to have similar chemical properties." (Ans. 3-4) In response to Appellants' argument that there is no evidence to establish a reasonable expectation of success in combining Gray with Luinstra (App. Br. 4), the Examiner argues that "[i]t is text book knowledge that hydrocarbyl groups (R) such as alkyl groups are much stronger organic bases compared to hydrocaryloxy groups such as alkoxides" and "[t]hus, one would have been motivated to replace the alkyl metal compound with the metal alkoxide since the metal alkoxide is easier and safer to handle and less expensive." (Ans. 4-5.)

Even assuming that the Examiner's "textbook knowledge" is correct, such knowledge, in and of itself, does not demonstrate that a person having ordinary skill in the art would have reasonably expected MR_2 and $M(OR^1)_2$ to be interchangeable in formulating Gray's Ziegler-Natta olefin catalyst component. Because the Examiner has not adequately explained why the disclosure of a monovalent Na complex as an intermediate for forming a catalyst for polymerizing formaldehyde demonstrates that $M(OR^1)_2$, where M is a Group II divalent metal, is interchangeable with MR_2 in the synthesis of a divalent Group II metal complex for use as a Ziegler-Natta catalyst, we cannot affirm." Ex Parte Coffy, 2008 WL 895743 *2 -*3 (Bd. Pat. App. & Interf.)(February 28, 2008)(Applicants' emphasis added).

"**The Examiner did not rely on any evidence demonstrating that a person of ordinary skill in the art would have reasonably expected that a friction welding step and a molten metal deposition step are interchangeable for Russell's purpose, which is to fill the spaces around the facing material bodies on the substrate in order to anchor the previously welded bodies firmly on the substrate**.

The fact that friction welding techniques are known, per se, (as demonstrated by Thomas and Talwar) does not demonstrate that a person having skill in the art would have reasonably expected a friction welding step to be interchangeable with the molten metal deposition step in Russell. Because the Examiner has not adequately explained why one of ordinary skill in the art would have reasonably expected that a friction welding step and a molten metal deposition step are interchangeable for Russell's purpose of filling in the spaces between the bodies welded to the substrate, we cannot affirm the rejections under consideration." Ex Parte Slattery, 2008 WL 838793 *5 (Bd. Pat. App. & Interf.)(March 4, 2008)(Applicants' emphasis added).

"Regarding the Examiner's second presented obviousness rejection of claims 13, 14, and 17-25 over Lindskog, Appellants contend that the Examiner has not presented a prima facie case of obviousness because Lindskog does not disclose or suggest an alloy that includes zirconium (Zr) as a constituent thereof (Br. 19-21). The Examiner counters this argument with the assertion that Lindskog discloses the presence of scandium (Sc), hafnium (Hf) and yittrium (Y) in the alloy and that one of ordinary skill in the art would have found it obvious at the time of the invention to replace at least some of the Hf with Zr given their

> common Group IVB status (Sc is a Group IIIB element) in the periodic table of elements (Ans. 14). **However, Appellants maintain that the Examiner has not provided persuasive evidence of the interchangeability of Zr with Hf for the alloy of Lindskog in support of this contention. Appellants argue that identifying elements as being in the same group of the periodic table, as the Examiner notes, does not establish their interchangeability for a specified alloy, especially given that elements in the same group of the periodic table typically have differing properties and are not generally known to be interchangeable on that basis alone (Reply Br. 12)**. Moreover, Appellants note that Lindskog suggests one or more lanthanides as an option (Reply Br. 12-13; Lindskog, col. 2, ll. 3-9). Zr is not a lanthanide series (rare earth) element.
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> **On this record, we agree with Appellants that the Examiner has not furnished a persuasive explanation as to how Lindskog, by itself, provides adequate evidence to establish a prima facie case of obviousness of the claimed subject matter. Accordingly, we reverse the Examiner's obviousness rejection over Lindskog.**" Ex Parte Hattendorf, 2008 WL 3276247 (Bd. Pat. App. & Interf.)(August 8, 2008)(Applicants' emphasis added).

As noted, Drummond does not even *mention* tin mesoporphyrin (or any other mesoporphyrin) therefore nothing that Drummond discloses or suggests regarding tin protoporphyrin would suggest to the artisan its *interchangeability* with tin mesoporphyrin for *any* purpose whatsoever. Additionally, Drummond itself notes that *in vitro* and *in vivo* behavior of a given *species* of metalloporphyrins (*i.e.* protoporphyrins) is fairly impossible to predict with accuracy when only the *metal* itself and not the *porphyrin* (proto- or meso-) is the variable:

> "**CONCLUSION**
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> **Metalloporhyrins in which the central iron atom of heme is replaced by other elements often display markedly different actions on heme metabolism *in vivo* from those of the inorganic metals they contain. In addition, the *in vitro* and *in vivo* actions of individual synthetic metalloporphyrins may also differ markedly, as exemplified by Co-protoporphyrin, which inhibits heme oxygenase activitiy *in vitro* but produces a sustained induction of the enzyme *in vivo*. The presence of the metal in the protoporphyrin macrocycle also serves to increase the extent and duration of action of the metal moiety on heme metabolism, and Co-protoporphyrin is a case in point. Contrasting effects on heme metabolism exist within the metallopophyrin species as demonstrated by the effects in the whole animal of Co-protoporphyrin and Sn-protoporphyrin on heme oxygenase activity—the former increasing enzyme activity, the latter inhibiting it. The recent demonstration that Sn-protoporphyrin, like Co-**

> **protoporphyrin, has dual actions on heme oxygenase activity *in vivo* (with the inhibitory action being expressed), confirms that synthetic metalloporphyrins possess both inductive and inhibitory properties for heme oxygenase and that the biological action that is ultimately expressed *in vivo* is dependent on the nature of the central metal atom of the metalloporphyrin**." (Drummond page 94; Applicants' emphasis added).

As regards Bettelheim, the Examiner's extrapolation of its limited disclosure of amino acids being "zwitterionic" to their all being "polar" and "water-soluble" is both inaccurate and irrelevant to the obviousness inquiry. First, it is well-known in the art that many amino acids are in fact *hydrophobic*. Indeed, the *hydrophobicity* of half of the amino acids recited in the Markush Groups of the instant dependent claims are noted in the art:

> "Ou *et al.* randomly mutagenized *mscL* in living bacteria and screened for mutants with leaky channels and hampered growth; it was found that most of the mutations in this group could be mapped to between residues 13 and 30, corresponding to one side of the TM1 helix. The authors infer from their data that this face of the helix moves from a hydrophobic to a hydrophilic region during gating, then back to a hydrophobic region. Interestingly, it was also observed that the mutation V23G results in a severe gain-of-function phenotype, though **glycine is hydrophobic** and smaller than valine. Thus, hydrophobic as well as specific packing interactions must play a role in MscL gating." (*See* page 2075 of Gullingsrud *et al.*, *Structural Determinants of MscL Gating Studied by Molecular Dynamics Simulations*, Biophysical Journal, Volume 80, May 2001, pages 2074–2081; hereinafter "Gullingsrud," attached hereto as **Exhibit D**, Applicants' emphasis added).

> "This conserved cysteine, located at amino acid position 84 in IDS, was replaced either by an alanine (C84A) or by a threonine (C84T). Cysteine and threonine are both polar amino acids, whereas **alanine is hydrophobic**. C84A and C84T mutant cDNAs were expressed in COS cells and in deleted Lβ. The C84A substitution had a drastic effect both for IDS processing and for catalytic activity. The C84T mutation produced a small amount of mature forms but also abolished the enzyme activity, confirming that the cysteine residue is required for IDS activity. MSD Lβ stably transfected with the wild-type IDS cDNA gave results similar to the C84T mutant. Previous expression studies of arylsulphatase A, arylsulphatase B and steroid sulphatase in MSD fibrobroblasts showed that polypeptides were normally processed but inactive, suggesting that a co- or post-translational modification of sulphatases is required for their catalytic activity [14]. In transfected MSD Lβ, IDS precursors were inactive, in contrast with IDS precursors secreted in the medium of overexpressing deleted Lβ that were catalytically active *in vitro* [4]. This result is in accordance with the hypothesis of

Schmidt et al.[5], who suggested that this modification occurs as an early posttranslational or co-translational event, most probably in the endoplasmic reticulum." (*See* page 247 of Millat *et al.*, *Characterization of iduronate sulphatase mutants affecting N-glycosylation sites and the cysteine-84 residue*, Biochem. J. (1997) 326, pages 243-247; hereinafter "Millat;" attached hereto as **Exhibit E**, Applicants' emphasis added).

"Our initial hypothesis was that the growth-inhibitory activity of the mutants G119R and E117L/G119R/A122D was associated with the increased amphipathicity of the third alpha helix. We have since developed evidence that the amphipathicity of the third alpha helix is largely irrelevant to that activity.

(1) The single E117L, like wt bGH, produced large animals.

(2) Mutant G119P produced the small animal phenotype even though proline is as hydrophilic as glycineT

(3) Mutant G119L produced the small animal phenotype even though **leucine is hydrophobic** and therefore disrupts the hydrophilic face of the helix.

(54) Mutant E111L/G119W/R125L produced the small animal phenotype even though all three mutations disrupt the hydrophilic face of the helix.

Thus, in one embodiment, the present invention relates to mutations of the third alpha helix which result in growth-inhibitory activity yet reduce or leave unchanged the amphiphilic character of the helix." (*See* page 26 of International Patent Application PCT/US90/05874, filed October 12, 1990 and published by WIPO as WO 91/05853 on May 2, 1991, naming as inventors Kopchick *et al.*, hereinafter "Kopchick;" attached hereto as **Exhibit F**, Applicants' emphasis added).

Indeed, even at its isoelectric point—the pH whereat Bettelheim notes all molecules of a given amino acid are in their zwitterionic form—phenylalanine is *hydrophobic*:

"**It is an important aspect of the process to maintain the pH in the range where the phenylalanine is present as a zwitterion and is hydrophobic**. At this pH, most of the other components present in the feed will be hydrophilic and will elute at the void volume. The preferred pH will be in the range of 4.5 to 6.5 with a pH of 6 being most preferred." (*See* col. 3, lines 17-24 of United States Patent 5,071,560, issued December 10, 1991 to McCulloch, *et al.*; hereinafter "McCulloch;" attached hereto as **Exhibit G**, Applicants' emphasis added).

And, even if the Examiner's mis-extrapolation of Bettelheim's *zwitterionic* teachings as being tantamount to a generality that *all* amino acids are water-soluble were *correct*, which it is **not**, the water-solubility *vel non* of any *given* amino acid (or of amino acids *generally*) is by no means a teaching or suggestion that the *complexing* of such an amino acid (or of amino acids *generally*) with tin mesoporphyrin would impart *solubility* to the insoluble tin mesoporphyrin.[3]

To be sure, the artisan would *only* appreciate those possibilities, and be guided to the instant claims, by considering the data and the teachings disclosed by the *instant* specification (*i.e.*, the *actually* reduced to practice fact of the esrtwhile insoluble tin mesoporphyrin *being made* soluble by Applicants' complexing of it with amino acids) in combination with the knowledge of one of ordinary skill in the art—a fact clearly stated in the specification itself:

> "**[0049]** **C)—Preparation of the Arginate Salt.** The tin (IV) mesoporphyrin IX dichloride prepared according to the process described above, is combined with an excess solution of arginine in aqueous sodium hydroxide (the ratio being about 2:1:3) and mixed for a sufficient period of time as to affect dissolution. The ratio of the tin (IV) mesoporphyrin IX dichloride to the arginine is about 2:1. The ratio of the tin mesoporphyrin IX dichloride to the aqueous sodium hydroxide is 1:3. The solution is then filtered, rinsed with deionized water and frozen. Following freezing of the solution, the frozen solution is vacuum dried to result in a lyophilized product.
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> **[0050]** We expect that the reconstituted product can be resolubilized into DI H_2O or 5% saline, or into one of other known in the art injectible or transdermal solutions, and delivered to the patient by such injectible or transdermal methods. **Those skilled in the art would readily appreciate that other amino acids would similarly react with tin (IV) mesoporphyrin IX dichloride to form a water soluble complex consistent with this invention.**
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> **[0051] For example, we have prepared and reacted a number of amino acids with tin-mesoporphyrin in the presence of NaOH. The material isolated from these reactions have been examined by ^{1}H NMR and UV/VIS (ultraviolet and visible) spectroscopy, as well as comparing the solubilities of the reaction products have also been compared to the solubilities of their**

[3] Additionally, Applicants note that each of the four above-noted-to-be-hydrophobic amino acids (glycine, alanine, leucine, and phenylalanine) are listed amongst the amino acids recited in Robinson's ¶[200], which ¶ is cited by the Examiner as a makeweight for the obviousness of making tin mesoporphyrin water-soluble through the utilization of amino acids.

> **respective starting materials, to determine whether an amino acid-tin-mesoporphyrin complex forms form such a reaction**." (*See* Instant Specification [0049] – [0051], Applicants emphasis added).

Accordingly, with regard to Bettelheim the instant rejection is a textbook example of hindsight reconstruction, masquerading behind a reference that does not stand for the proposition for which it is asserted (*i.e.*, amino acids are water-soluble, *therefore* anything complexed to an amino acid will be water soluble). And, in view of the above-noted *hydrophobicities* of various amino acids *themselves* the rejection fails to demonstrate any *reasonable expectation of success*, which, in the post-KSR world, is *still* required to be shown for purposes of establishing the obviousness of a claim to a combination:

> "A patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. *KSR,* 127 S.Ct. at 1741. **To demonstrate that a patent is invalid for obviousness based on a combination of references, "the burden falls on the patent challenger to show by clear and convincing evidence that a person of ordinary skill in the art would have had reason to attempt to make the composition ... and would have had a reasonable expectation of success in doing so.**" *PharmaStem Therapeutics, Inc. v. ViaCell, Inc.,* 491 F.3d 1342, 1360 (Fed.Cir.2007)." Depomed, Inc. v. Ivax Corp., 2007 WL 4365476 (N.D. Cal. 2007)(emphasis added).

In light of the foregoing, Applicants submit that independent claims 49, 52, 56, 60, 63, and 67 are nonobvious in view of the cited references. The remaining claims, each of which depends from claims 49, 52, 56, 60, 63, or 67 are each directed to further elements of the invention in combination with those of claims 49, 52, 56, 60, 63, or 67 and are therefore also patentable over the references. Applicants further submit that the application is in condition for allowance, and they therefore request its prompt passage thereto.

A cheque to cover the fee required by 37 C.F.R. §1.16(h), for the presentation of 3

additional independent claims in excess of three not already paid for, is herewith-included. It is believed that no further fee is due. However, if any further fee is due it should be charged to Deposit Account No.: 03-0678. Similarly, any credit for overpayment should be credited to Deposit Account No.: 03-0678.

CERTIFICATE OF MAILING

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8/21/09

Raymond E. Stauffer, Esq. **Date**

#371968 v1 - Response to Final Rejection

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residue

Residue

A residue in normal English speech is a product, usually unwanted, left behind by any process.In mathematics:In complex analysis, the residue is a complex number which describes the behavior of line integrals of a meromorphic function around a singularity.In modular arithmetic, the residue of an integer n to base b is the remainder r after the largest multiple mb of b no greater than n has been subtracted from n. (If n < 0, one adds multiples of b just sufficient to make the result non-negative.) The residues modulo (to the base) b form a ring.In a locally ringed space (X, OX), the residue of a section f in OX(U) at a point p of X is the reduction of the germ of f at p modulo the unique maximal ideal of the stalk of OX at p. When OX is a sheaf of functions on X, the residue of a section f at a point p is the value f (p).In general chemistry, a residue is what is left behind by a reaction.In chemistry a portion of a larger molecule is often called a residue. See residue (chemistry).In petroleum refining the residue comprises the heavier fractions that fail to vaporize. It can be used as fuel oil or cracked to produce lighter fractions. The volume and degree of cracking carried out is determined by the price difference between the light and heavy fractions and the cost of the cracking process. Crop residue
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residue
Noun
1. matter that remains after something has been removed
(hypernym) substance, matter
(hyponym) ash
2. something left after other parts have been taken away; "there was no remainder"; "he threw away the rest"; "he took what he wanted and I got the balance"
(synonym) remainder, balance, residual, residuum, rest
(hypernym) part, portion, component part, component
(hyponym) leftover, remnant

Related terms: husks, rest, vestige

Babylon Italian English dictionary Download this dictionary

residuo
adj. remaining, residual, residuary

Webster's Revised Unabridged Dictionary (1913) Download this dictionary

Residue
(n.)
That which remains of a molecule after the removal of a portion of its constituents; hence, an atom or group regarded as a portion of a molecule; -- used as nearly equivalent to radical, but in a more general sense.

(n.)
That which remains after a part is taken, separated, removed, or designated; remnant; remainder.

(n.)
That part of a testeator's estate wwhich is not disposed of in his will by particular and special legacies and devises, and which remains after payment of debts and legacies.

(n.)
Any positive or negative number that differs from a given number by a multiple of a given modulus; thus, if 7 is the modulus, and 9 the given number, the numbers -5, 2, 16, 23, etc., are residues.

Webster's Revised Unabridged Dictionary (1913), edited by Noah Porter. About

residue in Chinese | | residue in French | residue in Italian | residue in Spanish | residue in Dutch | residue in Portuguese | residue in German | residue in Russian | residue in Japanese | residue in Greek | residue in Korean | residue in Turkish | residue in Hebrew | residue in Arabic | residue in Croatian | residue in Serbian | residue in Swedish

Complex (chemistry)

This article is about the chemical complex. For other uses of this word, see complex.

A **complex** in chemistry and biochemistry is a reversible association of molecules, atoms, or ions through weak non-covalent chemical bonds. Simple salts are usually not considered complexes.

Contents [hide]

Metal complexes

A metal complex, also known as coordination compound, is a structure composed of a central metal atom or ion, generally a cation, surrounded by a number of negatively charged ions or neutral molecules possessing lone pairs.

The ions or molecules surrounding the metal are called ligands. A ligand that is bound to a metal ion is said to be **coordinated** with the ion. The process of binding to the metal ion with more than one coordination site per ligand is called chelation. Compounds that bind avidly to form complexes are thus called **chelating agents** (for example, EDTA).

Simple ligands like water or chlorine form only one link with the central atom and are said to be monodentate. Some ligands are capable of forming multiple links to the same metal atom, and are described as bidentate, tridentate etc. EDTA is hexadentate, which accounts for the great stability of many of its complexes.

Typically, the chemistry of complexes is dominated by interactions between s and p orbitals of the ligands and the d (or f) orbitals of the metal ions. Because of this, the simple octet rule fails in the case of complexes, and to understand the chemistry of these systems, a deeper understanding of chemical bonding rules is necessary.

One such rule is called electron counting, or the rule of 18. Crystal field theory, introduced by Hans Bethe in 1929, is a more quantum mechanically based attempt at understanding complexes. But crystal field theory treats all interactions in a complex as ionic. Ligand field theory, introduced in 1935 and built from molecular orbital theory, can handle a broader range of complexes and can explain complexes in which the interactions are covalent. The chemical applications of group theory can aid in the understanding of crystal or ligand field theory, by allowing simple, symmetry based solutions to the formal equations.

Naming complexes

The basic procedure for naming a complex:

1. Write the names of the ligands in alphabetical order.
2. *Multiply occurring monodentate ligands receive a prefix according to the number of occurrences: *di-*, *tri-*, *tetra-*, *penta-*, or *hexa*. Polydentate ligands (e.g., ethylenediamine, oxalate) receive *bis-*, *tris-*, *tetrakis-*, etc.
3. *Anions end in *o*. This replaces the final 'e' when the anion ends with '-ate', e.g. *sulfate* becomes *sulfato*. It replaces 'ide': *cyanide* becomes *cyano*.
4. *Neutral ligands are given their usual name, with some exceptions: NH_3 becomes *ammine*; H_2O becomes *aqua*; CO becomes *carbonyl*.
5. Write the name of the central atom/ion. If the complex is an anion, the central atom's name will end in *-ate*, and its Latin name will be used if available (except for mercury).
6. If the central atom's oxidation state needs to be specified (when it is one of several possible, or zero), write it as a Roman numeral (or 0) in parentheses.

Examples:

$[NiCl_4]^{2-}$ → tetrachloronickelate (II) ion

$[CuNH_3Cl_5]^{3-}$ → amminepentachlorocuprate(II) ion

$[Cd(en)_2(CN)_2]$ → dicyanobisethylenediaminecadmium(II)

Transition metals make good central ions for complexes.

To study the activity of complexes in solution, it is possible to record pH spectra which shows the interaction between complexing agent and central ion as a function of the degree of dissociation of their functional groups.

Receptor-ligand complexes

Receptors are proteins that bind small ligands. A typical example of a receptor-ligand complex is a neurotransmitter bound to a neurotransmitter receptor in the cell membrane of the synapse. The dissociation constant K_d is used as indicator of the affinity of the ligand to the receptor.

See also

- Dissociation (chemistry)

External links

Index of pH-spectra

KARGER

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Original Paper

Comparative Pharmacology of Zinc Mesoporphyrin and Tin Mesoporphyrin: Toxic Actions of Zinc Mesoporphyrin on Hematopoiesis and Progenitor Cell Mobilization

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Key Words

- Toxicity
- Zinc mesoporphyrin
- Tin mesoporphyrin
- Hematopoiesis
- Bone marrow
- Mobilization

Abstract

The effects of two synthetic heme analogues, zinc mesoporphyrin (ZnMP) and tin mesoporphyrin (SnMP), on in vivo hematopoietic progenitor cell mobilization and in vitro hematopoiesis were examined in rabbit bone marrow. Rabbits received granulocyte colony-stimulating factor (rhG-CSF) for 7 days in order to mobilize increased numbers of erythroid (BFU-E) and myeloid (CFU-GM) progenitors in peripheral blood. Concurrent treatment of rhG-CSF-treated rabbits with ZnMP reduced mobilization of the numbers of BFU-E (76% inhibition, $p < 0.0001$) and CFU-GM (70% inhibition, $p < 0.005$) in peripheral blood. In contrast, SnMP administered at the same concentration had no significant suppressive effect on BFU-E and CFU-GM recruitment. Both metalloporphyrins inhibited bone marrow heme oxygenase activity equally in vivo, thus indicating that both compounds enter bone marrow cells. Direct in vitro addition of ZnMP to normal rabbit bone marrow cultures suppressed BFU-E and CFU-GM growth, whereas SnMP had no such effect. These results confirm, in an in vivo system, our earlier in vitro studies and demonstrate that, at the concentrations studied, ZnMP, in contrast to SnMP,

displays toxicity for hematopoietic growth and progenitor cell production.

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Structural Determinants of MscL Gating Studied by Molecular Dynamics Simulations

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ABSTRACT The mechanosensitive channel of large conductance (MscL) in prokaryotes plays a crucial role in exocytosis as well as in the response to osmotic downshock. The channel can be gated by tension in the membrane bilayer. The determination of functionally important residues in MscL, patch-clamp studies of pressure-conductance relationships, and the recently elucidated crystal structure of MscL from *Mycobacterium tuberculosis* have guided the search for the mechanism of MscL gating. Here, we present a molecular dynamics study of the MscL protein embedded in a fully hydrated POPC bilayer. Simulations totaling 3 ns in length were carried out under conditions of constant temperature and pressure using periodic boundary conditions and full electrostatics. The protein remained in the closed state corresponding to the crystal structure, as evidenced by its impermeability to water. Analysis of equilibrium fluctuations showed that the protein was least mobile in the narrowest part of the channel. The gating process was investigated through simulations of the bare protein under conditions of constant surface tension. Under a range of conditions, the transmembrane helices flattened as the pore widened. Implications for the gating mechanism in light of these and experimental results are discussed.

INTRODUCTION

Mechanosensitive (MS) channels play an important physiological role in living cells of diverse phylogenetic origin. They are ubiquitous in prokaryotes and have recently been characterized in archaebacteria (Le Dain et al., 1998) as well as mammals (Patel et al., 1998; Maingret et al., 1999). In eukaryotes, MS channels play a role in such important biological functions as hearing, touch, and cardiovascular regulation (Corey and Hudspeth, 1983). In bacteria, response to the osmolality of their environment is essential for maintaining viability of the cell. In *Escherichia coli,* three MS channels have been identified, and one of these, MscL, has been cloned (Sukharev et al., 1994). Several studies (Blount et al., 1997; Ou et al., 1998; Ajouz et al., 1998) have confirmed the importance of this channel for osmoregulation of the bacterial cell. A bacterial cell exposed to osmotic downshock experiences an increase in cell membrane tension, which can lead to cell lysis unless the osmotic gradient can be relieved. In these circumstances, MS channels gate to allow K^+ and other osmoprotectants to be excreted from the cell. In eukaryotes, the stimulation of exocytosis by mechanical strain is thought to be mediated by stretch-activated channels (Xu et al., 1996; Weber et al., 2000).

The MscL protein exhibits a high degree of primary sequence conservation within a group of bacteria that includes *E. coli,* on which most physiology experiments have been performed, as well as *Mycobaterium tuberculosis,* from which the crystal structure was obtained. The determination of the crystal structure of MscL (Chang et al., 1998) revealed a protein with a homopentameric structure, approximately 50 Å wide in the plane of the membrane and 85 Å tall. Each 151-residue subunit consists of two transmembrane helices, labeled TM1 and TM2, and a cytoplasmic helix that extends some 35 Å below the membrane. The TM1 helices are arranged so as to block diffusion through the channel at their N-terminal ends; this region of the protein also exhibits very high sequence conservation. A loop region between TM1 and TM2 extends into the pore, which may also contribute to the conductance of the channel. Excision of the cytoplasmic domains has been found to have little effect on the gating properties of the channel (Ajouz et al., 2000).

In a series of patch-clamp experiments, Sukharev et al. (1999) characterized the response of MscL from *E. coli* to tension applied to reconstituted membranes. Conductance measurements indicated that MscL forms a pore at least 30 Å across. Single channel measurements revealed the existence of at least five subconductance states; the rate-limiting step in gating was found to be the transition between the closed state and the first subconductance state, with a barrier of 38 k_BT when no tension is applied. This first transition is also the only tension-sensitive part of the gating process; the free energies of the higher conducting states appear to be insensitive to tension. The authors conclude from their measurements that during the initial application of tension, the in-plane area of *E. coli* MscL increases greatly without a concomitant increase in conductance; subsequent internal rearrangements give rise to the subconductance states seen in patch-clamp measurements.

Yoshimura et al. (1999) studied the effect of mutating Gly22 to all other natural amino acids. It was found that both the growth rate of mutants as well as the threshold pressure of activation varied directly with the hydrophobic-

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0006-3495/01/05/2074/08 $2.00

ity of the substitution, with particularly acute growth inhibition from acidic substitutions.

Ou et al. (1998) randomly mutagenized *mscL* in living bacteria and screened for mutants with leaky channels and hampered growth; it was found that most of the mutations in this group could be mapped to between residues 13 and 30, corresponding to one side of the TM1 helix. The authors infer from their data that this face of the helix moves from a hydrophobic to a hydrophilic region during gating, then back to a hydrophobic region. Interestingly, it was also observed that the mutation V23G results in a severe gain-of-function phenotype, though glycine is hydrophobic and smaller than valine. Thus, hydrophobic as well as specific packing interactions must play a role in MscL gating.

Knowledge of the dynamics of MscL, e.g., of its thermal fluctuations, within a lipid bilayer environment would help to evaluate models that have been suggested for the gating of MscL by membrane tension, and may even suggest new mechanisms. Molecular dynamics simulations can give a detailed picture of the dynamics of MscL on the time scale of a few nanoseconds. The effect of surface tension can also be incorporated into molecular dynamics simulations. Examination of specific protein-lipid interactions will shed light on how MscL is able to gate by membrane tension alone. In such simulations, one can rationalize the results of mutagenesis experiments based on the environment observed around the residues. The overall rigidity or flexibility of the protein may give us some insight into the gating mechanism of MscL. Finally, the response of the protein to surface tension may give one clues as to how the protein responds to mechanical stress.

METHODS

Initial coordinates for the protein were taken from the crystal structure of Chang et al. (1998) (PDB entry 1msl). Residues following Glu104 were excised from the structure; these residues correspond to the C-terminus cytoplasmic helices and have been shown to be nonessential for channel gating and function (Ajouz et al., 2000). Residues 1–9, which were disordered in the crystal structure, were not modeled. The remaining structure (residues 10–104) was modeled using X-PLOR (Brünger, 1988) to place atoms that were absent from the crystal structure and to remove bad contacts.

The membrane used to provide the lipid environment for the protein was constructed from a palmitoyl-oleoyl-phosphatidylcholine (POPC) membrane taken from Heller and coworkers' 1993 molecular dynamics simulation (Heller et al., 1993). The system consisted of an equilibrated rectangular bilayer with 100 POPC lipids in each leaflet. Beginning from this structure, we performed a series of modeling steps in order to prepare a membrane suitable for our protein system. First, a narrow strip of lipids and water along the short side of the membrane was cut off and manually positioned on the long side of the membrane, making the membrane more square. After energy minimization to remove bad contacts, this new structure was equilibrated at 1 atm and 340 K for 1 ns, using the same methodology as described below, i.e., full electrostatics, periodic boundary conditions, and a flexible unit cell. This equilibrated membrane was too small to adequately contain the MscL structure. We constructed a larger membrane patch by replicating the equilibrated structure four times. The completely built protein structure was manually positioned in the membrane; overlapping water and lipid molecules were subsequently removed. After insertion, the protein and a square patch of membrane and water measuring 88 Å on each side was cut out of the large replicated system.

A pre-equilibrated water box was overlaid on the protein-lipid system in order to hydrate completely the aqueous part of the protein structure. Five chloride ions replaced five water molecules to bring the entire system to charge neutrality. The complete system was comprised of 7370 protein atoms, 195 POPC lipids, 7387 water molecules, and 5 chloride ions for a total of 55,666 atoms.

Molecular dynamics simulations were carried out using the program NAMD2 (Kale et al., 1999), with v.26 of the CHARMM force field (MacKerell et al., 1998) for proteins (MacKerell et al., 1992) and lipids (Schlenkrich et al., 1996). Bonds to all hydrogen atoms were kept rigid using SHAKE (Ryckaert et al., 1977), permitting a time step of 2 fs. The system was simulated in periodic boundary conditions, with full electrostatics computed using the particle mesh Ewald (PME) method (Darden et al., 1993) with a grid spacing on the order of 1 Å or less.

The system was energy minimized using the Powell algorithm, then heated for 2 ps under Langevin dynamics at a temperature of 310 K and with a damping coefficient γ of 10 ps^{-1}. The system was then equilibrated for 1 ns at constant pressure and temperature. Pressure was maintained at 1 atm using the Langevin piston method (Feller et al., 1995), with a piston period of 200 fs, a damping time constant of 100 fs, and piston temperature of 310 K. Temperature coupling was enforced by velocity reassignment every 2 ps.

Finally, the system was simulated for an additional 2 ns in the NpT ensemble using Langevin dynamics/Langevin piston at a temperature of 310 K and a damping coefficient of 10 ps^{-1}. Coordinates during this phase of the simulation were saved every picosecond.

Simulations were also performed with the same protein structure as above, but with no membrane or water. The Langevin piston method (Feller et al., 1995) was used to control the applied surface tension. Target values for the three Cartesian components of the pressure tensor were set using the formula (Chiu et al., 1995; Feller and Pastor, 1999)

$$P_x = P_y = P_z - \gamma/L_z$$

Here P_x, P_y, and P_z are the x, y, and z diagonal components of the pressure tensor and L_z is the size of the unit cell along the z axis, which is perpendicular to the plane of the membrane. The positions and momenta of the atoms in the protein were thereby coupled to changes in the area of the protein through the equation of motion of the Langevin piston. Simulations were performed with periodic boundary conditions, and with a cutoff of 14 Å for non-bonded interactions. Use of PME for the simulation of the bare protein was not warranted, given the qualitative nature of the model; moreover, the rapidly changing size of the dimensions of the unit cell would have made the accuracy of the electrostatics calculation degrade over time.

Analysis of trajectories and energetics was performed using X-PLOR (Brünger, 1992), VMD (Humphrey et al., 1996), and Matlab (MathWorks, Natick, MA).

RESULTS AND DISCUSSION

The system at the beginning of equilibration is shown in Fig. 1. At the beginning of the simulation, the protein was well immersed in the membrane, with no significant gaps between membrane and protein. The membrane patch was sufficiently large to accommodate MscL, whose function depends sensitively on its lipid environment. Throughout this article, we refer to TM1 as residues 15 to 43 of the protein, and TM2 as residues 69 to 89, following the previous nomenclature (Chang et al., 1998).

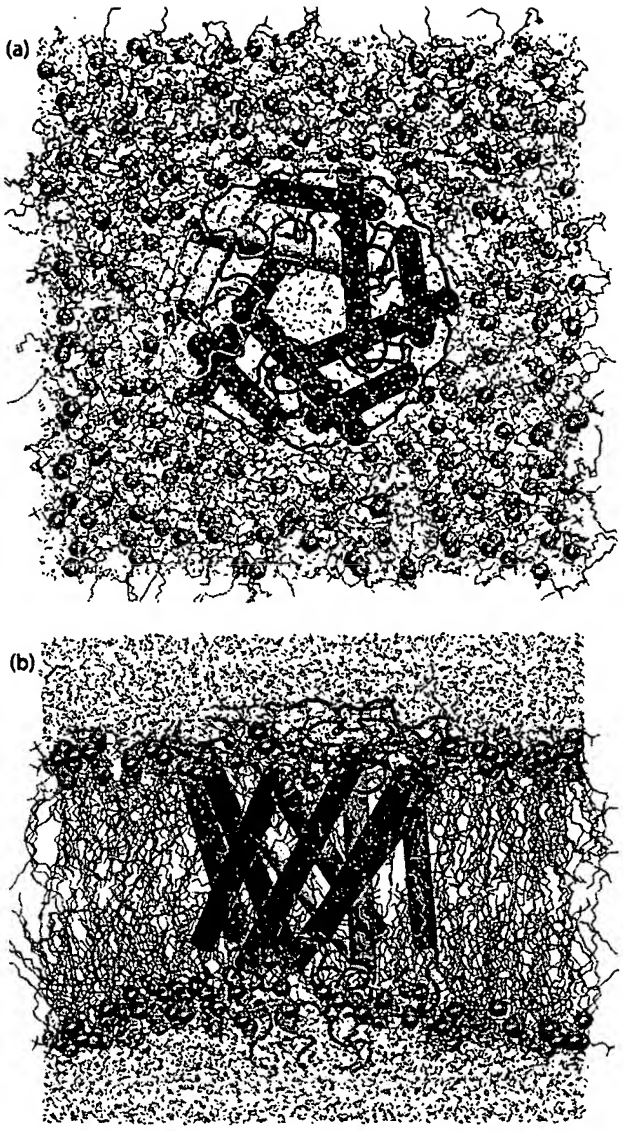

FIGURE 1 (*a*) Top view and (*b*) side view of MscL embedded in a POPC membrane at the start of the simulation.

Stability and fluctuations

Stability of the simulated protein can be assessed by analyzing the deviation of the structure from the initial crystal structure. Fig. 2 shows the root mean square deviation (RMSD) of the C_α atoms of the protein during equilibration and analysis. Preparation and heating of the protein are not shown in the figure; hence the RMS begins at ~2 Å at t = 0. As expected, the transmembrane segments of the protein are considerably more stable than the protein as a whole, which contains loop regions on each side of the membrane that extend into the solvent. It is evident from Fig. 2 that the transmembrane helices are quite stable by the time Langevin dynamics is begun at t = 1 ns. The RMSD during the dynamics period, ~2.5 Å, is comparable to the value obtained in other simulations of transmembrane helices (Forrest et al., 2000; Randa et al., 1999) as well as to that obtained for KcsA (Berneche and Roux, 2000; Shrivastava and Sansom, 2000).

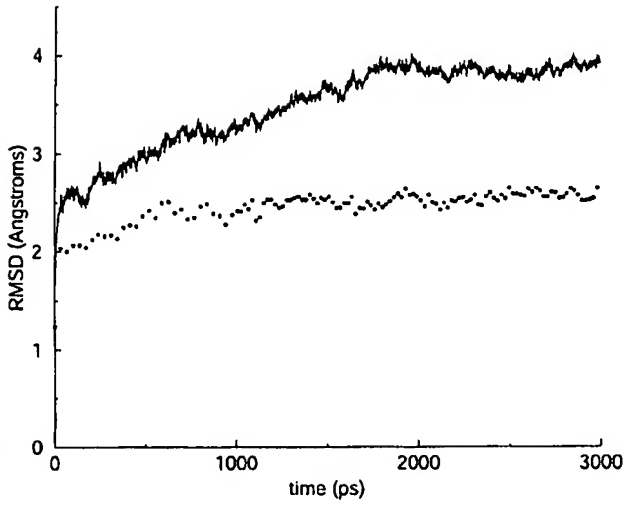

FIGURE 2 RMSD of C_α atoms during the simulation relative to the crystal structure. *Solid line:* all C_α atoms; *dotted line:* only C_α atoms in the transmembrane helices (residues 15–43 and 69–89). Overall center-of-mass translation of all transmembrane C_α atoms was removed before calculation of the RMSD.

Fluctuations about the mean positions indicate which parts of the system are most mobile. Fig. 3 shows snapshots

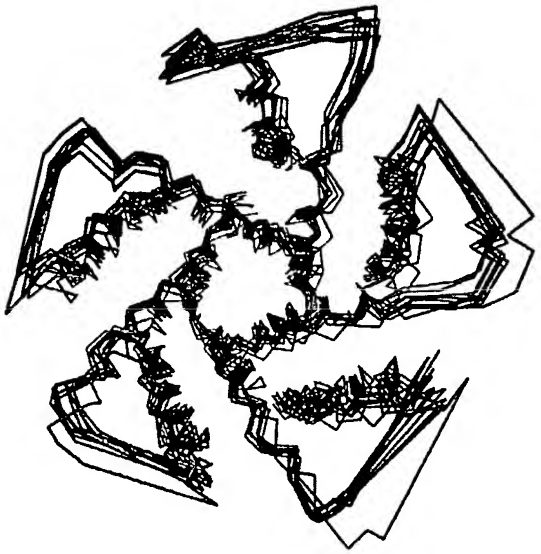

FIGURE 3 Backbone trace at 200-ps intervals during the last 2 ns of simulation.

of a trace of the backbone atoms during the last 2 ns of simulation. It is evident from the figure that the loop regions of the protein, corresponding to residues 46–68, are the most mobile part of the system, whereas the transmembrane helices are quite stable, both in terms of orientation and in terms of secondary structure.

Fig. 4 shows the RMS fluctuations for the C_α atom of each helix residue for TM1 and TM2 separately. The data show that the least mobile part of the protein corresponds to the first 4 to 5 residues of TM1, which pinch together to form a non-leaky occlusion. The TM2 helices show no such pattern; one of the TM2 helices moves as a rigid body away from its initial position, accounting for its large RMS values. The immobility of this part of the protein is in qualitative agreement with corresponding electron spin resonance experiments (G. E. Perozo and B. Martinac, personal communication).

Analysis of key residues

It is possible that secondary structure formation in the extracellular loop regions of the protein could affect the conductance of the channel. For this reason we examined hydrogen bond formation between backbone atoms in this region of the protein. Three pairs of residues were involved in hydrogen bond formation in at least two of the five subunits: Ile59-Ile67, Ile61-Ile65, and Ile67-Val48. In the case of Ile61-Ile65, the hydrogen bond formed between the backbone oxygen atom of Ile61 in one subunit and to the amide hydrogen of the neighboring subunit. These hydrogen bonds exhibited varying degrees of stability, as shown in Fig. 5.

We focused particular attention on a set of hydrogen bonds formed between Arg45 and Gln51 of neighboring

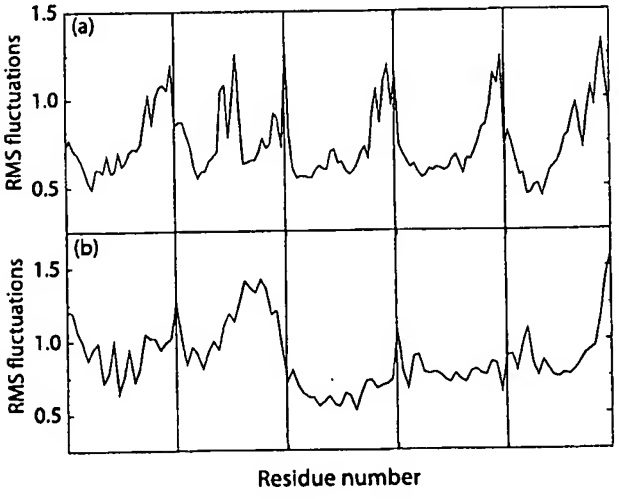

FIGURE 4 RMS fluctuations for C_α atoms in (*a*) TM1 and (*b*) TM2.

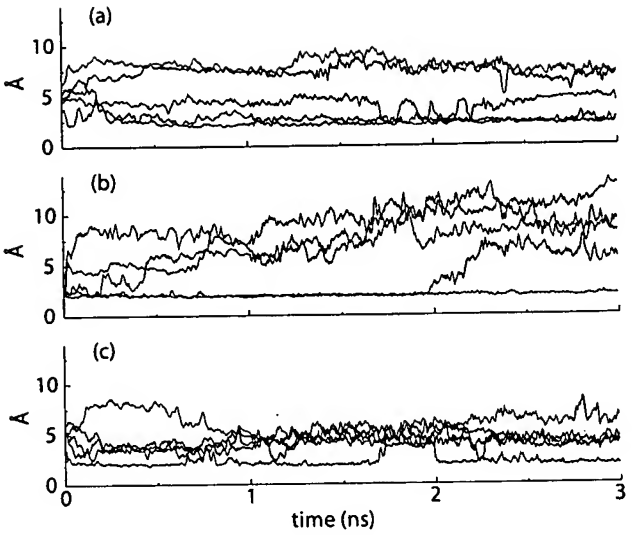

FIGURE 5 Backbone hydrogen bonding in the loop region of MscL; the first residue of the pair is the acceptor, the second is the donor. Distances shown are between the oxygen of the acceptor and the hydrogen of the donor. (*a*) Ile59-Ile67, (*b*) Ile67-Ile48, (*c*) Ile61-Ile65; in the last case, the two residues belong to neighboring subunits. Data shown are running averages of 20 data points, sampled at 1-ps intervals.

subunits, as shown in Fig. 6. Interaction between these residues has been shown (Maurer et al., 2000) to have a strong effect on MscL gating; when cysteine cross-links are introduced via mutagenesis at these sites, a gain-of-function mutant results. Fig. 6 shows the degree to which these two residues remained in close proximity during the 3-ns simulation. Two pairs of residues interact strongly during the entire simulation period, while a third appears to be drifting toward such an interaction and may have formed a long-lasting hydrogen bond as well. The loop regions in the other two subunits have unfolded too much for the bonds to have a chance to form.

We also examined interactions between transmembrane helices, both within subunits and in neighboring subunits. We found only one set of interactions that appeared with any stability in more than one subunit: the interaction between the side chain of Lys33 and the side chains of Ser74 and Asn78 of the neighboring subunit. Fig. 7 shows the distance between these residues during the 3-ns simulation, and a representation of the hydrogen-bonding network formed by these three residues. It is clear from Fig. 7 that at all times, both residues interact strongly with one or more hydrogen atoms of Lys33.

Penetration of water into the pore region of the protein is a key component of this analysis, in that the hydrophobicity of residues lining the pore has been shown to be critical for proper gating of the channel. Fig. 8 shows the extent to which the water molecules penetrated the pore at the end of the simulation. From the extracellular side of the pore, water molecules reach only to Thr25. Residues deeper in the

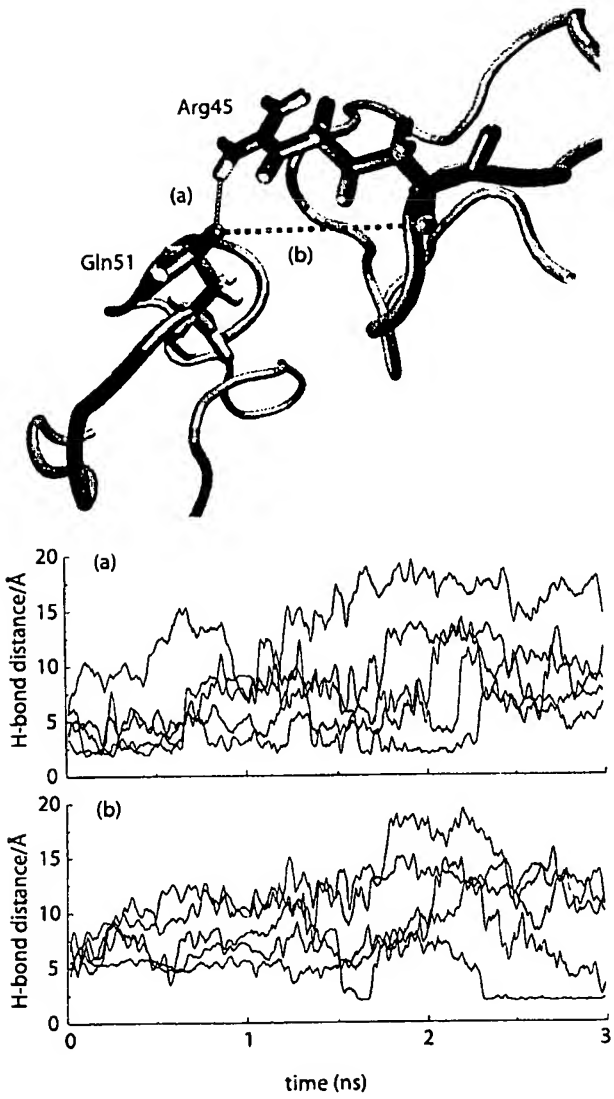

FIGURE 6 Hydrogen bonding between Arg45 and Gln51 of neighboring subunits. (*a*) Distance between glutamine oxygen and sidechain oxygen of Arg45 (see Fig. 7). (*b*) Distance between glutamine oxygen and amide hydrogen of Arg45. Data shown are running averages of 20 data points, sampled at 1-ps intervals.

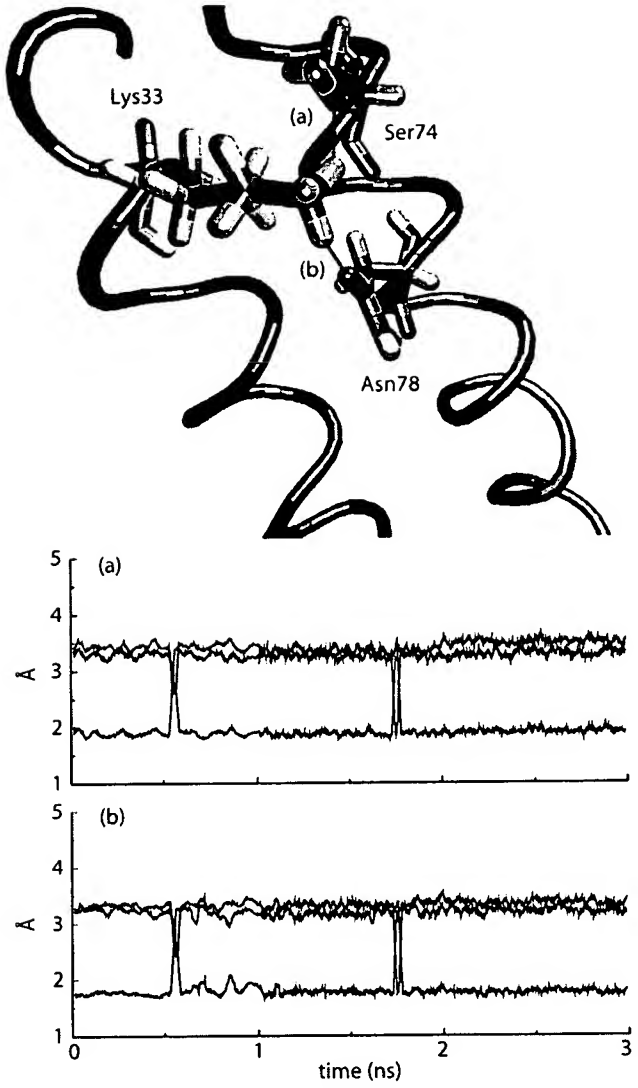

FIGURE 7 Inter-subunit hydrogen bonds between Lys33 and (*a*) Ser74 and (*b*) Asn78. Data shown are running averages of 20 data points, sampled at 1-ps intervals.

gating region, including Ala20 and Val21, are not exposed to water. The interfacial region near the intracellular side of the protein contains a number of water molecules, but these do not appear to have affected the stability of the pore.

Simulation of bare protein under surface tension

Though a realistic simulation of MscL must include the membrane and surrounding water, we can investigate the mechanics of the protein itself without these external media. To this end, we conducted a series of simulations of the same protein structure as in the membrane simulations, but with no membrane or water present. The simulations were conducted at constant surface tension and zero normal pressure.

Several factors influenced our choice of surface tension for these simulations. The value must be large enough to provoke a conformational change in the protein before the protein becomes unstable due to its unnatural environment; however, the surface tension must not be so large as to stretch the protein excessively. We ran a series of simulations with a normal pressure of 0 atm and a surface tension in the range of 10–200 dyn/cm. Previous membrane simulations (Chiu et al., 1995; Feller and Pastor, 1999) have suggested that a surface tension of 10–50 dyn/cm gives the best agreement with the measured lipid density of the mem-

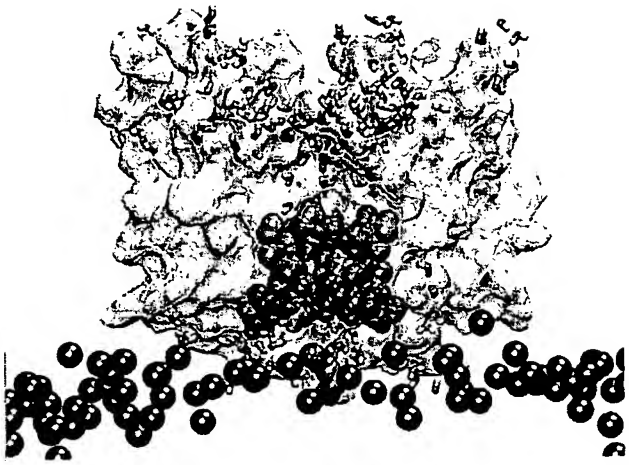

FIGURE 8 Water penetration in the pore at the conclusion of the simulation. Solid surface represents protein residues 15 to 40 and 69 to 89. Water molecules within 3 Å of TM1 residues are shown. Ala20 is shown in blue, Val21 in tan, and Thr25 in purple. Phosphate atoms in the intracellular leaflet of the membrane are shown for reference as gray spheres.

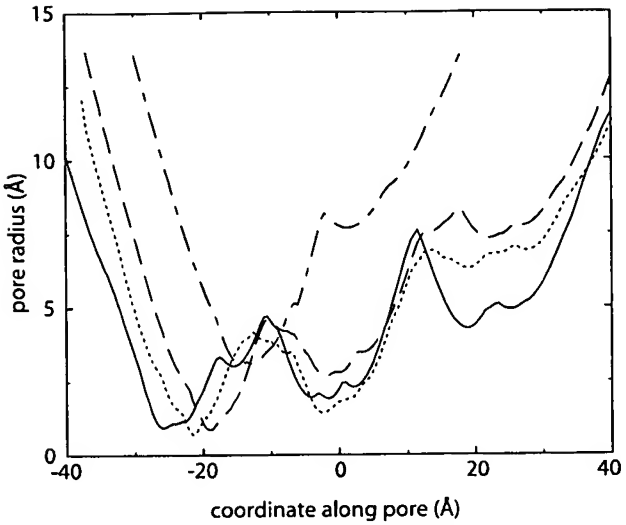

FIGURE 9 Radius of MscL pore as a function of the position along the pore axis, as calculated by the program HOLE, at four points in the simulation of the bare protein. *Solid line:* $t = 0$ ps; *dotted line:* $t = 50$ ps; *dashed line:* $t = 100$ ps; *dot-dashed line:* $t = 113$ ps. Protein coordinates at times later than $t = 0$ were fitted to the coordinates at $t = 0$ using the positions of the C_α atoms in TM1 and TM2.

brane. It should be emphasized here that our intention was to induce a non-equilibrium conformation change in the protein, while biasing as little as possible the pathway taken by the protein. Under all conditions studied, the protein refolded into an open conformation with minimal loss of secondary structure. The most visible difference between the simulations was the rate at which the protein refolded; this rate was nearly inversely proportional to the applied surface tension.

We describe here one representative simulation carried out with a surface tension of 60 dyn/cm. Analysis was performed for the first 115 ps, after which the rescaling introduced by the constant pressure method caused unphysical large changes in the protein structure.

Fig. 9 shows the radius of the MscL pore during the applied surface tension simulation, computed using the program HOLE (Smart et al., 1993). In the closed state of the channel, and in the snapshots at 50 and 100 ps, there were two primary points of constriction in the channel. Val103 and Glu104 formed the narrowest constriction, and Val21 and Thr25 formed a second constriction at the end of the extracellular pore. A third point of constriction was formed by the extracellular loops comprising residues 44–68.

During the first 100 ps of simulation with applied surface tension, the extracellular loops retracted from the center of the pore, resulting in the expansion of the pore radius by about 4 Å at $z = 10$–30 Å. The total in-plane area of the protein, as well as the angle formed by transmembrane helices with the membrane normal, remained essentially unchanged. During the next 13 ps, a dramatic shift in the tertiary structure of the protein took place. Both TM1 and TM2 helices tilted downward, producing a shortening of the total length of the pore. Val21 and Thr25 moved apart to allow water molecules to diffuse through this part of the channel. The total in-plane area of the protein increased from ~2300 Å^2 to 4100 Å^2. Val103 and Glu104 were pulled up toward what would be the interior of the membrane as the TM2 helices tilted downward; this is seen in the shift of the minimum pore radius in Fig. 9. Though these residues were sufficiently far apart to allow diffusion of water and ions, they still diminished significantly the total conductance of the channel. Fig. 10 illustrates the conformational changes associated with the gating during this simulation.

CONCLUSIONS

We have described the first molecular dynamics simulations of a representative of an important and ubiquitous class of proteins, the mechanosensitive ion channels. These simulations were only recently made possible by the determination of the crystal structure of MscL. Our simulations proceeded along two avenues of investigation: first, we thought to explain the nature of the protein in the closed state when placed in a realistic membrane and water environment; second, we asked how the protein would react to application of surface tension. Though it might still prove possible to investigate both questions with a single simulation, for reasons of computational efficiency we sought to divide our efforts between two different approaches.

Results of the simulations of the protein in the full membrane-water system reveal a protein that is quite stable in the closed state. This is to be expected from patch-clamp data

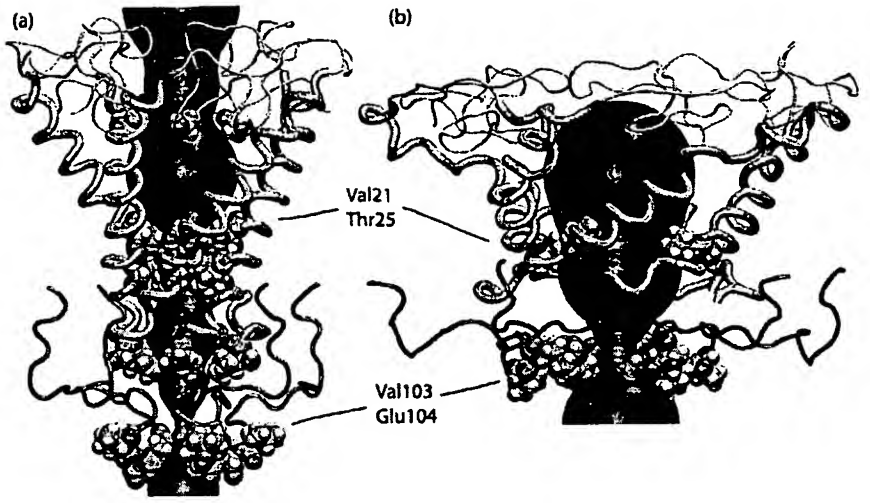

FIGURE 10 MscL pore structure at (*a*) t = 0 and (*b*) t = 113 ps. The solid blue region in each picture corresponds to pore radius as calculated by the program HOLE. Pink tubes correspond to TM1 residues, white tubes represent the extracellular loop region (residues 44–68), and purple tubes correspond to residues 89–104. In both pictures, residues Val21, Thr25, Val103, and Glu104 are shown in space-filling representation; (*a*) also shows Ile14 near the bottom of the figure and Gly63 near the top. TM2 helices are omitted for clarity.

(Sukharev et al., 1999), which reveal a channel that is absolutely nonleaky until significant tension is applied. Large-scale changes in the shape of the protein could not be expected during the progress of a 3-ns simulation; it is possible, therefore, that a much longer simulation could reveal a somewhat different closed state. We believe that we have described the essential features of this protein on the time scale of several nanoseconds, and find encouraging correspondence with experiments. Fluctuations on the scale of individual residues were found to be in good agreement with corresponding measurements from electron spin resonance experiments, confirming the validity of our protein model. Water penetration in the pore was found to extend only to hydrophilic residues, i.e., only as far as Thr25. This result lends support to proposed mechanisms of MscL gating that postulate a change in the solvent environment of hydrophobic residues in the constricted region of the protein during gating.

Our simulations of the bare protein using an applied surface tension to induce conformational change provided remarkably consistent results: the protein retained its secondary structure while radically reforming its tertiary structure to form a large pore. Retention of secondary structure was an important validity check, since the native lipid environment would not have allowed alternative hydrogen bonds to form. The observation that the transmembrane helices flattened out corresponds well with recent measurements made of the effect of membrane thickness on MscL gating (Kloda and Martinac, 2001). In these measurements, it was found that when MscL was placed in a thinner membrane, it remained in its open state for a longer period. This would seem to suggest that the open conformation of MscL is flatter than the closed structure. The simulation of the bare protein also suggested a role for particular sets of residues in the gating process. Val21 and Thr25 formed a tight constriction at the end of the extracellular pore, which was abolished only after significant tilting of the transmembrane helices. Val103 and Glu104 retained a constricted arrangement even after the rest of the pore expanded. It remains to be seen whether these observations remain valid in a fully hydrated environment; the absence of water in the pore could have contributed to a decrease in stability of the constriction formed by Val21. The interactions between MscL and the surrounding bilayer are also quite complex, and we are currently pursuing simulations in which tension is applied to a complete protein-membrane-water system.

Molecular dynamics simulations of membrane protein systems are becoming increasingly common even as the systems' sizes continue to grow (Lin and Baumgaertner, 2000; Berneche and Roux, 2000; Shrivastava and Sansom, 2000). Our simulations were of comparable duration and of slightly larger size than other related studies; this reflects in part our desire to construct a membrane large enough to encapsulate the protein fully within a realistic environment without the effects of boundary conditions. There is accumulating evidence (Chiu et al., 1995; Feller and Pastor, 1999) that surface tension plays an important role in obtaining the most realistic membrane structure. Though a number of investigators have simulated helices and proteins embedded in membranes without the use of a nonzero surface tension and obtained apparently reasonable results, we expect to see more simulations in which surface tension is controlled. Due to the large size of thermodynamic fluctuations in two-dimensional systems such as membranes, and due to the slow time scales in which lipid diffusion takes place, a substantial computational effort must be made in order to pursue these investigations.

In the case of MscL, we have even more reason to look closely at the role of surface tension. Our simulations of MscL under the influence of surface tension are to our knowledge the first application of this type of external force to study conformational changes in proteins. Our work on

MscL will continue with constant surface tension simulations of the protein embedded in the membrane-water system.

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Characterization of iduronate sulphatase mutants affecting N-glycosylation sites and the cysteine-84 residue

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Iduronate sulphatase (IDS) is responsible for mucopolysaccharidosis type II, a rare recessive X-linked lysosomal storage disease. The aim of this work was to evaluate the functional importance of each N-glycosylation site, and of the cysteine-84 residue. IDS mutant cDNAs, lacking one of the eight potential N-glycosylation sites, were expressed in COS cells. Although each of the potential sites was used, none of the eight glycosylation sites appeared to be essential for lysosomal targeting. Another important sulphatase co- or post-translational modification for generating catalytic activity involves the conversion of a cysteine residue surrounded by a conserved sequence C-X-P-S-R into a 2-amino-3-oxopropionic acid residue [Schmidt, Selmer, Ingendoh and von Figura (1995) Cell **82**, 271–278]. This conserved cysteine, located at amino acid position 84 in IDS, was replaced either by an alanine (C84A) or by a threonine (C84T) using site-directed mutagenesis. C84A and C84T mutant cDNAs were expressed either in COS cells or in human lymphoblastoid cells deleted for the IDS gene. C84A had a drastic effect both for IDS processing and for catalytic activity. The C84T mutation produced a small amount of mature forms but also abolished enzyme activity, confirming that the cysteine residue at position 84 is required for IDS activity.

INTRODUCTION

Iduronate sulphatase (IDS; EC 3.1.6.13; L-iduronate-2-sulphate 2-sulphohydrolase) is one of the lysosomal enzymes involved in the degradation of heparan sulphate and dermatan sulphate. IDS deficiency is responsible for mucopolysaccharidosis type II, a rare X-linked lysosomal storage disease [1]. A 2.3 kb cDNA clone containing the complete coding region of IDS was isolated, and the reading frame contains 550 amino acids [2]. Normal IDS processing has been studied after IDS cDNA overexpression and endocytosis in fibroblasts [3], and the processing steps were found to be identical in COS cells and in lymphoblastoid cell lines (Lβ) [4]. In our study, the functional importance of each N-glycosylation site and of the conserved cysteine-84 residue was evaluated.

Sequence analysis of IDS cDNA revealed the presence of eight potential N-linked glycosylation sites (Asn-X-Ser/Thr) at positions 31, 115, 144, 246, 280, 325, 513 and 537 (Figure 1). Eight IDS mutant cDNAs lacking selected N-glycosylation sites were prepared by site-directed mutagenesis and expressed in COS-7 cells. The processing of these mutants was studied and their catalytic activity measured to evaluate the functional importance of each of these glycosylation sites.

Another potentially important co- or post-translational modification for all sulphatases is the conversion of a conserved cysteine residue into a 2-amino-3-oxopropionic acid residue. The deficiency of this conversion has been demonstrated for arylsulphatase A in MSD (multiple sulphatase deficiency) fibroblasts [5]. Using site-directed mutagenesis, the conserved cysteine-84 of IDS was replaced by either an alanine (C84A) or a threonine (C84T). These two mutants IDS cDNAs were expressed either in COS-7 cells or in lymphoblastoid cells and analysed for processing and catalytic activity. IDS processing and activity were also studied in the MSD Lβ stably transfected with the normal IDS cDNA.

MATERIALS AND METHODS

Materials

Tritiated disaccharide substrate O-(α-L-idopyranosyluronic acid 2-sulphate)-(1 → 4)-2,5-anhydro-D-[^{3}H]mannitol 6-sulphate was purchased from Professor Krasnopolskaya (University of Mos-

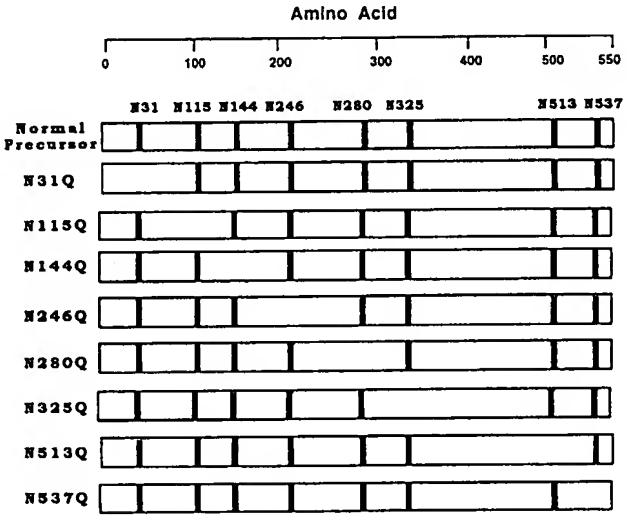

Figure 1 Construction of IDS cDNA lacking individual potential glycosylation sites

Positions of the eight glycosylation sites (N31, N115, N144, N246, N280, N325, N513, N537) are indicated. Eight mutant cDNAs (N31Q, N115Q, N144Q, N246Q, N280Q, N325Q, N513Q, N537Q) lacking one potential glycosylation site were constructed.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EndoH, endoglycosidase H; IDS, iduronate sulphatase; Lβ, lymphoblastoid cell line; MSD, multiple sulphatase deficiency; PNGaseF, *N*-glycosidase F.

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Table 1 Oligonucleotides used for mutagenesis

The altered codons are indicated in bold, italic characters.

Oligonucleotides	Sequences
N31Q	5′-GAAACGCAGGCC***CAG***TCGACCACAGAT-3′
N115Q	5′-GTGCACGCTGGA***CAG***TTCTCCACCATC-3′
N144Q	5′-GGGATATCTTCT***CAG***CATACCGATGAT-3′
N246Q	5′-TATCCCTTGGAG***CAG***ATCACCCTCGCC-3′
N280Q	5′-GTCCAAGCCTTA***CAG***ATCAGTGTGCCG-3′
N325Q	5′-CTTCAGCTGGCC***CAG***AGCACCATCATT-3′
N513Q	5′-GAATTTCTAGCT***CAG***TTTTCTGACATC-3′
N537Q	5′-CACAATATGTAT***CAA***GATTCCCAAGGT-3′
C84A	5′-CGCAGCAAGCAGTG***GCC***GCCCCGAGCCGCG-3′
C84T	5′-CGCAGCAAGCAGTG***ACC***GCCCCGAGCCGCG-3′
MUT-*Xho*I	5′-GCTCGACGGTACTCGCGGAACTGAAAAACC-3′

cow, Russia). The full-length IDS cDNA clone (pB2Sc17) was kindly provided by Professor J. J. Hopwood (Adelaide Children's Hospital, North Adelaide, South Australia 5006, Australia). U.S.E. mutagenesis kit and Protein A–Sepharose CL-4B were from Pharmacia. pTK vector was from Clontech. Dulbecco's modified Eagle's medium (DMEM)/25 mM Hepes was from Gibco. PMSF and tunicamycin were from Sigma. Reagents for SDS/PAGE and the Gene Pulser electroporation unit were from Bio-Rad. Sequenase Quick-Denature Plasmid Sequencing Kit, Amplify, and Rainbow ^{14}C-labelled methylated protein molecular-mass markers were from Amersham. Tran ^{35}S label and DMEM deficient in methionine and cysteine were from ICN. Endoglycosidase H (EndoH) and *N*-glycosidase F (PNGaseF) were from BioLabs.

Cell lines

Deleted Lβs were from a mucopolysaccharidosis-type-II patient presenting a complete deletion of the IDS gene [6].

MSD Lβs were from a patient affected by MSD. The biochemical diagnosis was assessed by demonstrating the deficiency of several sulphatases in skin fibroblasts: arylsulphatases A and B, iduronate-2-sulphatase, heparan sulphamidase, *N*-acetylglucosamine-6-sulphate sulphatase and steroid sulphatase.

Lβ and COS cells were cultivated in RPMI 1640 and in DMEM/25 mM Hepes respectively supplemented with 12% (v/v) fetal-calf serum and antibiotics at 37 °C in a humidified air/CO_2 (19:1) incubator.

Transfection procedure

Lβ were electroporated to enhance transfection with a eukaryotic vector containing the full-length human IDS cDNA under the control of the Rous Sarcoma Virus (RSV) promoter (pREP-IDS) [7]. The experimental conditions were: 5×10^6 cells in 500 μl of RPMI 1640/25 mM Hepes, 50 μg of plasmid DNA (pREP-IDS) and a voltage pulse of 250 V, 960 μF. At 48 h post-electroporation, the cells were selected with 50 units/ml hygromycine B. Resistant cell lines were maintained in complete RPMI 1640 medium containing 50 units/ml hygromycine B.

COS cells were electroporated to enhance transfection with a eukaryotic vector containing the normal (pTK-IDS) or mutated IDS cDNA under the control of the thymidine kinase promoter. The experimental conditions were: 20×10^6 cells in 500 μl of DMEM/25 mM Hepes, 100 μg of plasmid DNA and a voltage pulse of 220 V, 960 μF.

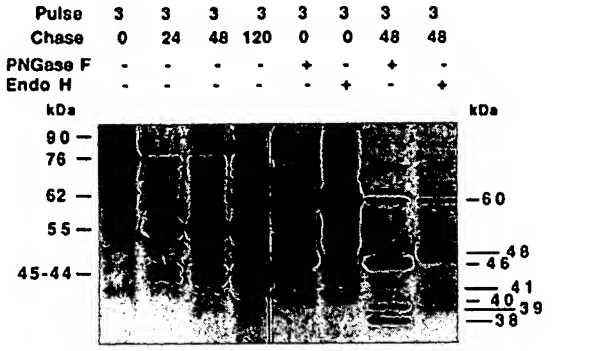


Figure 2 Digestion of IDS polypeptides by EndoH or PNGaseF in transfected COS cells

COS cells transfected with wild-type cDNA were labelled for 3 h (pulse) and harvested directly or after the indicated chase period. IDS polypeptides were immunoprecipitated from cell homogenates, incubated with either EndoH or PNGaseF, and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left and the right.

Constructs

IDS mutants were generated by site-directed mutagenesis using the U.S.E. mutagenesis kit (Pharmacia). Each mutant was constructed using one single-stranded vector (pTK-IDS or pREP-IDS) and two oligonucleotides: one mutagenic oligonucleotide containing the desired mutation and the MUT-*Xho*I oligonucleotide to abolish the unique *Xho*I restriction site in the vector for selection of mutated plasmids. The sequences of these oligonucleotides are indicated in Table 1. For each of the eight glycosylation sites, the asparagine codon (Asn) was converted into a glutamine codon (Gln). The mutated plasmids were sequenced by the dideoxy chain-termination method [8].

Pulse–chase labelling, immunoprecipitation, glycosidase digestions and IDS activity measurements

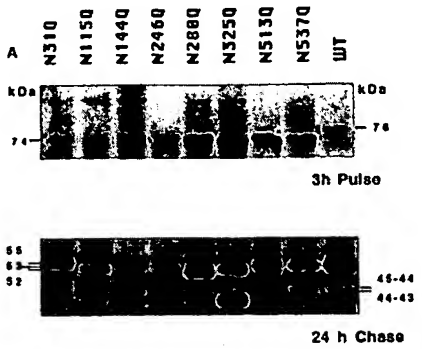
Experimental conditions have been previously described [3].

RESULTS

Functional characterization of IDS N-glycosylation sites

Glycosylation studies (Figure 2)

COS cells transfected with the wild-type cDNA produced a 76 kDa precursor that was rapidly converted into a smeary phosphorylated 90 kDa form (3 h pulse). The 90 kDa precursor is converted into a major intermediate 62 kDa form giving the 55 kDa (major form) and 45–44 kDa (doublet) mature polypeptides (24 h chase). These ^{35}S-labelled IDS polypeptides were digested by either EndoH or PNGaseF. After EndoH or PNGaseF digestion, the 76 kDa band was converted into a unique 60 kDa band: this precursor contains only hybrid and/or high-mannose-type oligosaccharide chains. After digestion by EndoH, the smeary 90 kDa band was converted into a band with a slightly lower molecular mass, while, after PNGaseF digestion, a unique 60 kDa band was observed: the 90 kDa precursor contains some hybrid and/or high-mannose glycans and mostly complex glycans. The 62 kDa intermediate and 55 kDa mature form were digested to a 48 kDa form by EndoH and to a



B

CONSTRUCTS	N31Q	N115Q	N144Q	N246Q	N280Q	N325Q	N513Q	N537Q	WT
CELLS (units)	7532	2090	1309	1239	4622	3832	2640	3868	3072
MEDIA (units)	786	324	276	138	2160	708	240	330	390
SECRETED %	9.5	13.4	17.4	10.0	31.8	15.6	8.3	7.9	11.3

Figure 3 IDS pulse–chase labelling and IDS activity in COS cells transfected with N-glycosylation mutant cDNAs

(A) COS cells transfected with wild-type (WT) and mutant cDNA constructs were labelled for 3 h (pulse) and harvested directly, or after a 24 h chase. IDS polypeptides were immunoprecipitated from cell homogenates and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left and the right. (B) COS cells were harvested 48 h after transfection. Cell extracts and media were assayed for IDS activity. One unit is defined as the amount of enzyme that releases 1 pmol of tritiated disaccharide substrate per hour. IDS activity values are the averages of three different transfections in which the basal activities of COS cells were subtracted.

46 kDa form by PNGaseF. The 45–44 kDa form was converted into a 41–40 kDa form after EndoH digestion, and into a 39–38 kDa form after PNGaseF digestion. These three IDS polypeptides contain both hybrid, high-mannose, and at least one complex-type, oligosaccharide chains.

Functional characterization of each N-glycosylation site (Figure 3)

^{35}S-labelled wild-type and mutant IDS polypeptides were immunoprecipitated from cell homogenates after a 3 h pulse period or after a subsequent chase of 24 h, and analysed by SDS/PAGE (Figure 3A). After a 3 h ^{35}S-labelling period, all mutant precursor forms showed a slightly lower molecular mass (74 kDa) than the wild-type precursor (76 kDa): each potential N-glycosylation site was glycosylated.

After a 24 h chase period, the 74 kDa precursor form of the N31Q, N513Q and N537Q IDS mutants were processed to the normal 55 kDa and 45–44 kDa mature forms (Figure 3A). N115Q, N144Q, N246Q, N280Q and N325Q IDS mutants were processed to lower-molecular-mass mature forms of 53 kDa and 44–43 kDa, but the ratio of the 43 kDa mature form to the 53 kDa mature forms was higher for the N325Q IDS cDNA mutant.

IDS activity was measured in cell extracts and in the media of transfected COS cells. Cells transfected either by the wild-type cDNA or by an N-glycosylation mutant showed a striking increase in IDS activity compared with the basal IDS activity of COS cells (150–200 pmol·h^{-1}·mg^{-1}) (Figure 3B). The lowest activities were observed for mutants N144Q and N246Q. In the media, the percentage of secreted IDS activity was similar in the wild-type and in the mutants, except the N280Q mutant, for which a 3-fold increase was observed.

Table 2 IDS activities in stably transfected Lβ

Cells	Constructs	IDS activity (pmol·h^{-1}·mg^{-1})
Deleted Lβ	C84A	0
Deleted Lβ	C84T	0
Deleted Lβ	Wild-type	5464
Deleted Lβ	Vector only	0
MSD Lβ	Wild-type	0
MSD Lβ	Vector only	0
Normal Lβ	Vector only	29

Table 3 IDS activities in transiently transfected COS cells

Cells	Constructs	IDS activity (pmol·h^{-1}·mg^{-1})
COS cells	C84A	169
COS cells	C84T	144
COS cells	Wild-type	3985
COS cells	Vector only	132

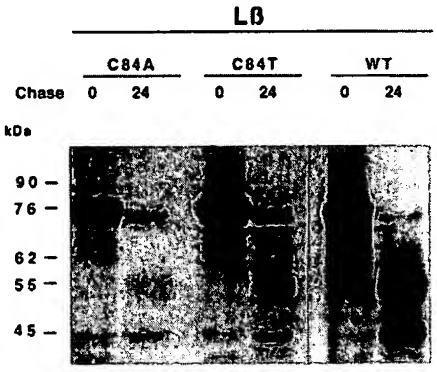


Figure 4 ^{35}S pulse–chase labelling in Lβ stably transfected with C84A or C84T mutant cDNAs

Lβ stably transfected with wild-type, C84A and C84T constructs were labelled for 3 h (pulse) and harvested directly or after a 24 h chase. IDS polypeptides were immunoprecipitated from cell homogenates and separated by SDS/PAGE. Exposure time was 6 days, except for cells transfected with the wild-type cDNA (2 days). Positions of molecular-mass markers are shown on the left.

Functional analysis of C84A and C84T IDS mutant cDNAs

Transfection of C84A and C84T IDS cDNA mutants in Lβ and in COS cells

After transfection of COS cells or Lβ with C84A or C84T IDS cDNAs, no significant increase in IDS activity could be observed, but after transfection with normal IDS cDNA, activity reached 30 times normal in COS cells and 200 times normal in Lβ (Tables 2 and 3).

The 76 kDa precursor produced in C84A Lβ was poorly converted into the 90 kDa form (pulse 3 h), leading to an

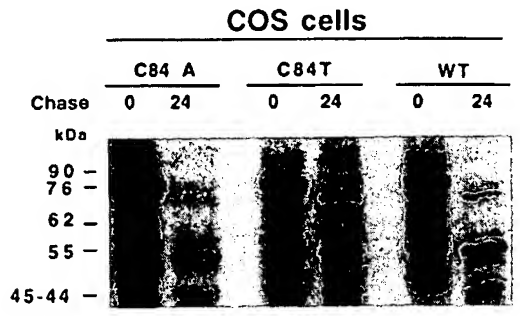


Figure 5 ^{35}S pulse–chase labelling in COS cells transiently transfected with C84A or C84T mutant cDNAs

COS cells transiently transfected with wild-type, C84A and C84T constructs were labelled for 3 h (pulse) and harvested directly or after a 24 h chase. IDS polypeptides were immunoprecipitated from the cell homogenates and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left.

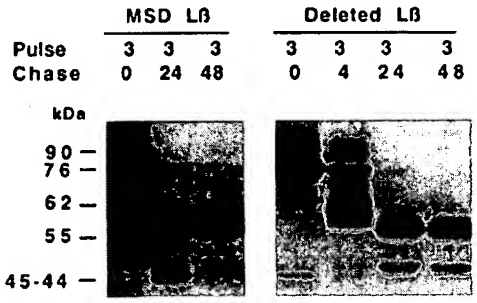


Figure 6 ^{35}S pulse–chase labelling of MSD Lβ and deleted Lβ stably transfected with the wild-type IDS cDNA

MSD and deleted Lβ stably transfected with wild-type cDNA were labelled for 3 h (pulse) and harvested directly or after the indicated chase period (h). IDS polypeptides were immunoprecipitated from cell homogenates and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left.

important decrease in the mature polypeptides produced (chase 24 h) (Figure 4). In Lβ stably transfected with the C84T cDNA, the amount of the 76 kDa precursor processed into the 90 kDa form was also decreased, but not as much as in C84A Lβ. Consequently, Lβ expressing C84T cDNA produced more mature forms than Lβ expressing C84A cDNA.

Similar results were observed in COS cells transiently transfected with either the C84A cDNA or the C84T cDNA (Figure 5).

Transfection of MSD Lβ with the normal IDS cDNA

In non-transfected MSD Lβ, no IDS activity could be measured even after a long-term culture. After transfection of MSD Lβ with the wild-type IDS cDNA, activity remained undetectable (Table 2). Pulse–chase experiments showed, after a 3 h ^{35}S-labelling period, the presence in normal amount of the 76 kDa precursor form poorly converted into 90 kDa forms (Figure 6). After a 24 or 48 h chase, the 90 kDa precursors were converted into 55 and 45 kDa mature forms.

DISCUSSION

IDS undergoes several post-translational modifications during its transport to lysosomes. The initial glycosylated 76 kDa precursor form is converted into a phosphorylated 90 kDa form (Scheme 1). This form is then processed by other glycosylation modifications and proteolytic cleavages through various intermediates to a major 55 kDa mature form. Further partial proteolytic cleavage gives the 45 kDa mature form.

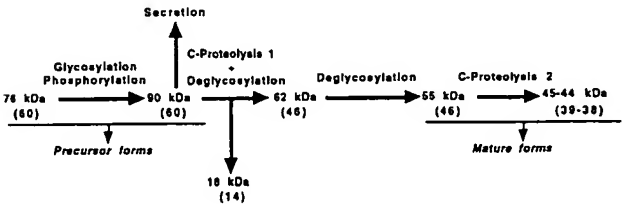
Functional characterization of IDS N-glycosylation sites

Asparagine residues in the sequence Asn-X-Ser/Thr (X = any amino acid but Pro) are the potential sites for attachment of N-linked oligosaccharide chains transferred *en bloc* from the dolichol pyrophosphate. Site-directed mutagenesis of each potential glycosylation site was used to establish the number of sites used and their functional role. A molecular-mass decrease of approx. 2 kDa (corresponding to the removal of one carbohydrate chain) was observed for all mutant precursor forms, showing that each potential N-glycosylation site was used. Pulse–chase experiments showed that all mutant precursors were correctly processed to the mature forms: none of the glycosylation sites was essential for lysosomal targeting of IDS. Similar results were reported for some lysosomal enzymes [9–11], but not for all of them: α-glucosidase was not processed if the second glycosylation site at Asn-233 was abolished [12].

IDS activity of cells transfected with the various mutants was close to that obtained with the wild-type cDNA. IDS activity secreted in the medium by the N280Q mutant was 3-fold higher than the wild-type: this suggests that this glycosylation site may be the most important for lysosomal targeting. A preferential phosphorylation of this glycosylated site could account for that result, as it has been suggested for β-glucuronidase [9]. The four N-glycosylation sites of β-glucuronidase were glycosylated, but the absence of the preferentially phosphorylated sites 2 and 3 resulted in very reduced amounts of the lysosomal form and increased secretion [9].

Study of IDS cDNA mutants lacking two or more glycosylation sites would probably result in a cumulative effect on processing and enzyme activity, as shown for β-glucuronidase [9]. Unglycosylated IDS precursors synthesized in the presence of tunicamycin were not processed and inactive, showing that N-glycosylation is required for processing and enzyme activity [4]. Unglycosylated precursors of lysosomal enzymes are probably misfolded and degraded in the endoplasmic reticulum [13].

Comparison of the mobility shifts of the mutant precursor and mature forms confirmed the localization of some of the proteolytic sites. Three IDS cDNA mutants (N31Q, N513Q or N537Q) synthesized the normal 55 kDa mature form: processing from 76 to 55 kDa involves an N-terminal proteolytic step



Scheme 1 IDS processing in COS cells

Molecular masses of deglycosylated polypeptides are indicated in parentheses (kDa).

downstream of Asn-31, and a C-terminal proteolytic step upstream of Asn-513. As previously described [2], the N-terminal proteolytic pre-lysosomal cleavage removes a propeptide of eight amino acids (amino acids 26–33) and occurs after the removal of the 25-amino-acid N-terminal signal sequence. The C-terminal proteolytic cleavage (Scheme 1) (C-proteolysis 1) produces the 62 kDa intermediate form and 18 kDa polypeptide [3]. The 18 kDa polypeptide has been previously sequenced [2] and the N-terminal identified at amino acid residue 456.

The further proteolytic step was the conversion of the 55 kDa mature form into the 45–44 kDa mature polypeptides. As indicated by the molecular mass of these deglycosylated forms, this second proteolytic step led to the release of a 7–8 kDa polypeptide (Scheme 1). This step is probably a C-terminal proteolytic cleavage (C-proteolysis 2), because the removal of 7–8 kDa from the N-terminal sequence of the 55 kDa form would eliminate the highly conserved sequence C^{84}-X-P-S-R^{88}.

Functional analysis of two site-directed mutations affecting Cys-84 of the IDS gene

Another important sulphatase co- or post-translational modification involves the conversion of a cysteine residue surrounded by a conserved sequence (C-X-P-S-R) into a 2-amino-3-oxopropionic acid residue [5]. Schmidt et al. [5] have proposed that this conversion of the cysteine is required for the catalytic activity of sulphatases. Its deficiency would be the cause of MSD, as structural analysis of arylsulphatase A and arylsulphatase B from MSD fibroblasts demonstrated that cysteine-to-serine semialdehyde conversion was deficient. In this rare autosomal recessive disorder, all sulphatase activities are severely decreased, despite a normal processing [14].

This conserved cysteine, located at amino acid position 84 in IDS, was replaced either by an alanine (C84A) or by a threonine (C84T). Cysteine and threonine are both polar amino acids, whereas alanine is hydrophobic. C84A and C84T mutant cDNAs were expressed in COS cells and in deleted Lβ. The C84A substitution had a drastic effect both for IDS processing and for catalytic activity. The C84T mutation produced a small amount of mature forms but also abolished the enzyme activity, confirming that the cysteine residue is required for IDS activity. MSD Lβ stably transfected with the wild-type IDS cDNA gave results similar to the C84T mutant. Previous expression studies of arylsulphatase A, arylsulphatase B and steroid sulphatase in MSD fibroblasts showed that polypeptides were normally processed but inactive, suggesting that a co- or post-translational modification of sulphatases is required for their catalytic activity [14]. In transfected MSD Lβ, IDS precursors were inactive, in contrast with IDS precursors secreted in the medium of overexpressing deleted Lβ that were catalytically active *in vitro* [4]. This result is in accordance with the hypothesis of Schmidt et al. [5], who suggested that this modification occurs as an early post-translational or co-translational event, most probably in the endoplasmic reticulum.

Previous studies have reported that the residual activities of arylsulphatases A, B and C vary in MSD fibroblasts and can be affected by culture conditions such as pH, medium composition and culture time [15]. A varying IDS activity ranging from 3% to 25% of mean normal value was measured in our patient's fibroblasts at different times (results not shown). In contrast, no IDS activity could be observed in untransfected and transfected Lβ from the same MSD patient (even after a 10-month culture), whereas arylsulphatases A and B showed a variable residual activity. These results suggest that the saturable mechanism of conversion varies from one cell type to another and has a greater affinity for arylsulphatases A and B than for IDS in Lβ.

The functional characterization of IDS mutants affecting the N-glycosylation sites and the cysteine-84 residue substantiated the knowledge of IDS maturation and confirmed, for IDS, the importance of the modification of the conserved cysteine for sulphatase activity.

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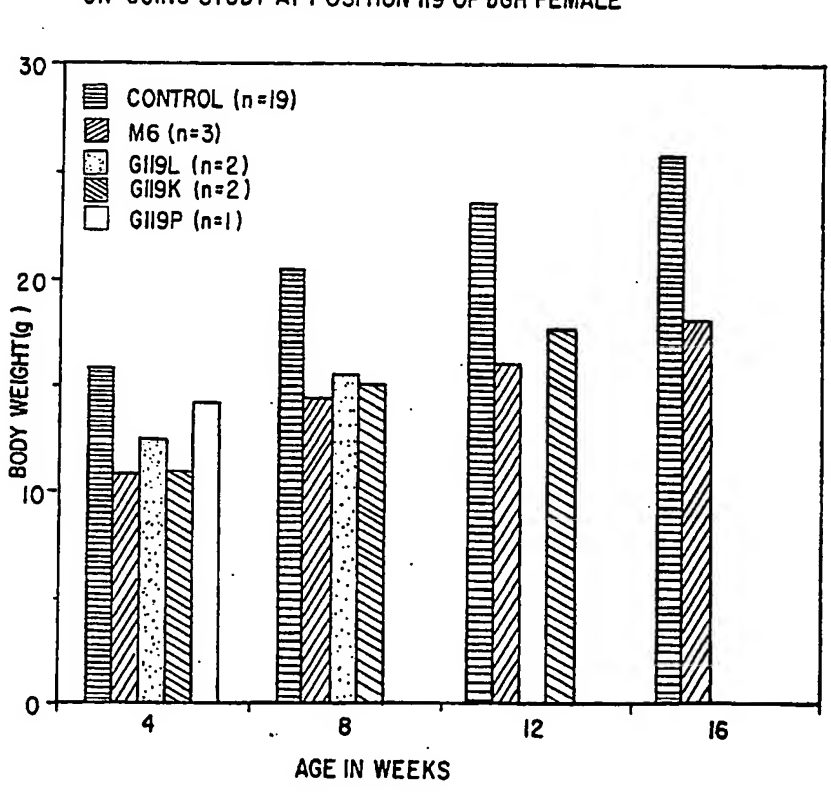
(54) Title: GROWTH HORMONE ANTAGONISTS

(57) Abstract

The present invention relates to growth inhibitory antagonists of bovine growth hormone obtained by mutation of the third alpha helix of that protein. These novel hormones may be administered exogenously to animals, or transgenic animals may be made that express the antagonist and thereby exhibited a reduced growth phenotype.



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GROWTH HORMONE ANTAGONISTS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to growth hormone, especially bovine growth hormone, muteins which inhibit the growth of animals. These analogues may be expressed in transgenic animals which thereby acquire a "reduced growth" phenotype.

Information Disclosure Statement

Bovine growth hormone (GH) is a protein of 191 amino acids that is naturally synthesized in the anterior pituitary. The molecular weight of the mature protein is about 22,000 daltons, but it is initially made as a pre-growth hormone with an extra 26 amino acids on the amino terminal. This leader (or signal peptide) is normally cleaved during secretion of the hormone by bovine pituitary cells. Several forms of the mature protein have been found in nature. The N-terminal can vary (due to variation in the site of cleavage during secretion) so that the mature protein begins with either NH_2-Ala-Phe-Pro or NH_2-Phe-Pro. Additionally, the amino acid at position 126 may be either leucine or valine, apparently as a result of allelic variation in the bovine population.

Exogenous administration of bGH to cattle increases milk production, feed efficiency, growth rate, and the lean-to-fat ratio, and decreases fattening time.

bGH has been produced by recombinant DNA techniques, see e.g., Fraser, U.S. 4,443,539 (yeast); Buell, EP Appl. 103,395 (bacteria); Krivl, EP Appl. 193,515 (bacteria); Kopchick, EP Appl. 161,640 (encapsulated mouse cells implanted into animals); DeBoer, EP Appl. 75,444 (bacteria; gene modified

to eliminate harmful secondary structure) and this has facilitated the production of analogues of bGH by site-specific mutagenesis. Thus, Aviv, GB 2,073,245 describes production of Met Pro (des Ala) bGH, Met Arg (des Ala) bGH, Met-Glu-Gly (des Ala) bGH, and des (Ala^1-Phe^2-Pro^3-Ala^4) bGH in E. coli. Brems, et al., PNAS (USA) 85:3367-71 (1988) reported preparation of the bGH mutant K112L, which extended the hydrophobic face of the third alpha helix of bGH. The 96-133 fragment of this mutant was also prepared. The biological activity of proteolytic fragments of bGH has also been studied. Brems, et al., Biochemistry, 26:7774 (1987); Swislocki, et al., Endocrinology, 87:900 (1970); Paladini, et al., TIBS, 256 (Nov. 1979). The fragment of bGH containing amino acids 96-133 is superior in growth promoting assays to bGH 1-95 and bGH 151-191. Hara, et al., Biochemistry, 17:550 (1978); Sonenberg, U.S. Patent Nos. 3,664,925 and 4,056,520; Chen and Sonenberg, J. Biol. Chem., 250:2510-14 (1977).

Similar results were observed with the fragment bGH (96-133). Graf, et al., Eur. J. Biochem., 64:333-340 (1976); Hara, et al., Biochem., 17:550-56 (1978). The information obtained through analyses of peptide fragments is of uncertain value since the native conformation of the molecule may be dramatically disrupted.

Analogues of bGH have varied in growth-promoting activity, as have the known analogues of other growth hormones. However, a growth hormone analogue having growth-inhibitory activity has not previously been reported.

A variety of transgenic animals have been produced. Hammer, et al., Nature, 315:680-683 (1985) (rabbits, sheep and pigs). Certain of these animals have been caused to express a growth hormone, and increased growth of such transgenic animals has been reported. Palmiter, et al., Nature 300:611 (1982) microinjected the male pronucleus of fertilized mouse eggs with a DNA fragment containing the promoter of the mouse

metallothionein-I gene fused to the structural gene of rat growth hormone. Several of the transgenic mice developed from the genetically modified zygote exhibited a growth rate substantially higher than that of control mice. (In effect, the genetically modified mouse serves as a test environment for determining the effect of the hormone on animal growth). Later, Palmiter, et al., Science, 222:809 (1983) demonstrated that a similar enhancement of growth could be obtained in transgenic mice bearing an expressible human growth hormone gene. A like effect is observed when human growth hormone releasing factor is expressed in transgenic mice. Hammer, et al., Nature, 315:413 (1985).

Bovine growth hormone has also been expressed in transgenic animals. McGrane, et al. J. Biol. Chem., 263:11443-51 (1988); Kopchick, et al., Brazil. J. Genetics, 12:37-54 (1989).

However, transgenic animals characterized by an exogenous gene which confers a reduced growth phenotype were hitherto unknown.

Researchers have attempted to predict the secondary structure of a protein from its primary amino acid sequence Chou and Fasman, Biochemistry, 13:222 (1974). The secondary structure of a polypeptide is a regular arrangement of a linear segment of the polypeptide chain. The most commonly encountered secondary structures are the beta-sheets and the alpha-helices. Helices may be described by the rise per element d, the number of elements per turn n, and the distance r of a marker point on each element (e.g., the C_α atom) from the helix axis. The mean values of these parameters for alpha helices in polypeptides are as follows:

n, 3.6 residues/tum,
d, 1.5 angstroms/residue, and
r, 2.3 angstroms.

See Schulz and Schimer, Principles of Protein Structure 69 (Springer-Verlag: 1979). The alpha helix is stabilized by hydrogen bonding between peptide amide and carbonyl groups of residues separated by a single turn of the helix.

Secondary structure predictions are based on observation of the frequency of occurrence of the amino acid in a beta-sheet, alpha-helix, etc. in a protein having a known three dimensional structure.

SUMMARY OF THE INVENTION

The present invention relates to proteins which are substantially homologous with a vertebrate growth hormone but have growth-inhibitory activity.

We have discovered that mutation of Gly^{119} in bGH to Arg ("G119R"), Pro ("G119P"), Lys ("G119K"), Trp ("G119W") and Leu ("G119L") and the mutation of Ala^{122} to Asp ("A122D") results in a mutein (mutant protein or peptide fragment thereof) which has growth-inhibitory activity in vertebrates, especially mammals. This novel hormone may be administered to mammals (or other vertebrates), in particular bovines, when growth inhibition is desirable. In one embodiment of the invention, the hormone is produced exogenously and administered to the subject. In view of the size of the hormone, it is preferably produced by expression in a suitable host of a gene coding for it. Such a gene is most readily prepared by site-specific mutagenesis of a bGH gene. However, the hormone may also be produced by other techniques, such as by condensation of fragments of native bGH with a synthetic peptide carrying the replacement amino acid.

In a second embodiment of the invention, this gene is introduced into a prenatal form of a mammal by known techniques, and the prenatal form is developed into a transgenic mammal which expresses a reduced growth phenotype. Conceivably, a mammal could be genetically modified after birth, i.e., "gene therapy".

Thus, growth-inhibited animals may be produced either by administration of the growth inhibitory hormone of this invention in pharmaceutical form, or by genetic transformation of a prenatal or postnatal form of the animal.

The hormone, or the gene encoding it, is useful in the production of small animals for use in research facilities where space is restricted, as pets for pet lovers with limited quarters, and as livestock for farmers having small tracts. The hormone may also be useful in the treatment of human giantism, and in research on giantism and dwarfism.

In the course of our work, we have discovered a correlation between the ability of mouse L cells to secrete the protein and the protein having an effect (positive or negative) on growth rate in a transgenic animal. The use of an L cell secretion assay to identify growth-modulating proteins is also a part of this invention.

The appended claims are hereby incorporated by reference as a further enumeration of the preferred embodiments. All patents and publications cited herein are incorporated by reference to the extent pertinent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Amino acid sequence of bGH (G119R) and nucleotide sequence of the gene encoding this analogue. The alpha helices are marked and the amino acids are numbered, with

number 1 being the first amino acid of the mature protein. The boldfaced bases and amino acids are those mutagenized in the G119R mutant.

Figure 2 General strategy of oligonucleotide-directed mutagenesis. pBGH10Δ6 was used as the parental vector. It contains mouse metallothionein I transcriptional regulatory sequences (MT-1) fused to the bGH gene (BamHI joint with BglII) which contains five exons (shaded boxes I-V) and intron A. This fusion gene was incorporated into pBR322 at the EcoRI site. The pBR322 origin of replication (ORI), ampicillin resistant gene (Amp), as well as the bGH translation start (ATG) and stop (TAG) codons are indicated. 5' and 3' non-translated regions are shown in hatching. The nucleotide sequence between restriction sites Tth111I and XmaI is shown. Substitution mutations are indicated. One silent situation is also indicated (*) which created a unique BamHI site. The position of the principal amino acid residues targeted for change (115, 117, 119, 122) are indicated.

Figure 3 is an idealized surface net (cylindrical plot) representation of most of the third alpha helix of bovine growth hormone. The surface net is produced by projection of the helix onto a coaxial cylindrical sheet of paper, cutting this paper parallel to the helical axis and flattening it. The volumes of the amino acids are given in parentheses. A dashed line indicates the cleft or depression formed by Ala122-Gly11a-Asp115.

Figure 4 is a plot of the secondary structure prediction (alpha-helix, beta-sheet, reverse turn, random coil) for amino acids 108-127 of bovine growth hormone (a) wild-type (b) the mutant G119R and (c) the mutant A122D. These plots were generated by the "Micro-Genie" program.

Figure 5 depicts a receptor binding assay, (wild-type) bGH versus mutant bGH G119R). This figure shows that the mutant bGH (G119R) has a greater affinity for the growth hormone receptor of mouse liver membrane preparations than wild type bGH.

Figure 6 provides a growth rate comparison among control (non-transgenic), G119R, G119L, G119K and G119P mice, illustrating the growth-inhibitory effect of these mutants.

Figure 7 presents an axial view of the third alpha helix (109-126) of bGH, showing its amphipathic tendencies. Hydrophobic amino acid sectors are shaded by dots; the glycine sector, a neutral amino acid, by slanted lines. The residue numbers and hydrophilicity values (Hopp and Wood scale) are given.

Figure 8 presents side views of the third alpha helix of wild type (left) and G119R mutant (right) bGHs projected on the plane in which the side chain of the Arginine-119 of the mutant G119R lies. The bottom of the cleft is indicated by an arrow.

The views were prepared by use of molecular modelling software (QUANTA and CHARMm, Polygene, Waltham, Massachusetts, USA).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The three-dimensional structure of porcine growth hormone has been determined by X-ray diffraction and compared to that of other growth hormones. Abdel-Meguid, et al., Proc. Nat. Acad. Sci., 84:6434 (1987). Like the other growth

hormones thus studied, it is a single domain protein arranged as a four helix bundle with the helices in an antiparallel relationship. Its four helixes are made up of residues 7-34, 75-87, 106-127 and 152-183.

Bovine growth hormone is 93% homologous at the amino acid sequence level with porcine growth hormone, and bGH's structure has been deduced by study of two sequences and of the structure of porcine growth hormone. Its four alpha helixes have been reported to be assumed by amino acids 4-33, 66-80, 108-127 and 150-179, though for purposes of the present invention, the third alpha helix of bGH is defined as amino acids 109-126. The conformation is reasonably consistent with the predictions made by Chen and Sonenberg, Biochemistry, 16:2110 (1977) using the method of Chou and Fasman, supra (10-34, 66-87, 111-127, 186-191).

The amino acid sequence of the growth hormones isolated from various vertebrate species are highly conserved. In a comparison of flounder growth hormone with other growth hormones, including bGH, Watahiki, et al., J. Biol. Chem., 264:312 (1989) identified five conserved regions. Watahiki's conserved region GD4 comprises the stretch LKDLEEGILALMRELED of bovine growth hormone, i.e., residues 113 to 129. Watahiki's Figure 3 identifies residues conserved among the GHs and residues predicted to be important for the manifestation of growth-promoting activity.

It has been shown that a recombinant molecule containing a hGH-(1-134) fragment linked to a human placental lactogen-(14-191) fragment retained full hGH immunological activity and binding affinity to GH receptors isolated from rabbit liver. Russell, et al., J. Biol. Chem., 256:296-300 (1981). By using the homolog-scanning mutagenesis technique, gene fragments of homologous hormones -i.e., human placental lactogen or human prolactin - were systematically substituted throughout the hGH gene, thus producing various

chimeric hormones. Cunningham, et al., Science, 243:1330-36 (1989). A comparison of the binding affinities of these mutants GHs and wild-type hGH to a cloned liver hGH receptor led to the conclusion that there were three discontinuous polypeptide determinants in hGH involved in receptor binding. They were located at the NH_2 terminus, COOH terminus, and within a loop between amino acid residues 54 and 74. These putative binding domains were further analyzed by an alanine-scanning mutagenesis technique in which alanine residues were systematically substituted throughout those regions. Amino acid residues at positions 10, 58, 64, 68, 172, 174, 175 and 176 of hGH were shown to be important for GH receptor binding. Cunningham, et al., Science, 244:1081-85 (1989).

The present invention relates to growth-inhibitory compounds (peptides or proteins) having a similarity in sequence and secondary structure to a vertebrate growth hormone, including but not limited to mammalian growth hormones, especially bovine growth hormone. Preferably, the compound comprises an alpha helix having an amino acid sequence homology of at least about 50% with the third alpha helix of a vertebrate growth hormone, especially bovine growth hormone. Other alpha helices of the native hormone may be omitted if this can be done without loss of growth-inhibitory activity.

The overall percentage homology of bovine growth hormone with other mammalian growth hormones is high: porcine (92%), ovine (99%), human (66%), and rat (87%). Insofar as the third alpha helix (amino acid sequence homologous to bGH 109-126) is concerned, the percentage homology is comparable to the overall figure: porcine (94%), ovine (94%), human (66%) and rate (94%).

The present invention is not limited to the mutation of the third alpha helix of bovine growth hormone. Rather, it encompasses the mutation of the third alpha helix of any mammalian or other vertebrate growth hormone, including, but

not limited to, the growth hormones whose sequences are given in Watahiki (1989): flounder, yellowtail, tuna, salmon, chicken, rat, porcine, ovine, bovine and human growth hormones. Expression of mutants of other growth hormones is facilitated by the availability of genes encoding the latter. See, e.g., Goeddel, Nature, 281:544-683 (1979) (hGH).

The concept of a polypeptide which is substantially homologous to bovine growth hormone is deemed to include (but is not limited to) any polypeptide which differs from bovine growth hormone by (a) a substitution at an amino acid corresponding to amino acids 115, 119 or 122 of bovine growth hormone, (b) a substitution at an amino acid corresponding to an amino acid of bovine growth hormone which is not conserved among the vertebrate growth hormones, and/or truncation of amino acids 1-95 and/or 134-191. (Conserved amino acids are identified in Watahiki, et al., 1979.) Thus, all non-bovine vertebrate growth hormones are "substantially homologous" with bovine growth hormone.

The compound is considered to be growth-inhibitory if the growth of test animals of at least one vertebrate species which are treated with the compound (or which have been genetically engineered to express it themselves) is significantly slower than the growth of control animals (the term "significant" being used in its statistical sense). Preferably, it is growth-inhibitory in a plurality of species. Growth hormones have considerable interspecies cross-reactivity. Gill, et al., Biotechnology, 3:643 (1985) reported that recombinant chicken and bovine growth hormones accelerate growth in juvenile pacific salmon.

It is known that certain fragments of growth hormones also have growth-promoting activity, and it is expected that the growth-inhibitory peptides (the term is used hereafter to include proteins) of the present invention need not be as large as bGH. Preferably, the peptides are at least 11 amino acids

long (three turns of an alpha helix) and more preferably at least 50 amino acids long. These peptides may retain the growth inhibiting action of, e.g., bGH (G119R), yet lack other, undesirable biological activities of the native size mutant. They may also have more desirable pharmacokinetic characteristics.

The growth inhibitory peptides of the present invention may also be larger than bGH, provided that the additional amino acids do not result in the compound being unable to reduce the growth rate of a vertebrate.

While the mechanism of action of applicant's growth-inhibitory peptides is not known, it is believed that they function as antagonists to wild-type growth hormones endogenously produced by the target animal. We have shown that, e.g., bGH (G119R) and bGH (G119R, E117L, A122D), both competitively inhibit the binding of wild type bGH to liver membrane preparations. Thus, it is believed that the compound has a net result of inhibiting growth because its growth-promoting activity is substantially less than that of wild type growth hormones (and perhaps is negligible) yet it can displace from growth hormone receptor sites the endogenous native growth hormone (whose stimulation of growth would have been more pronounced). However, applicants are not bound by this theory. Preferably, the compounds of the present invention have ED50 which is less than about 10 times the ED50 of wild type bGH in an assay of the ability of the compound to displace radiolabeled wild type bGH from a liver membrane preparation made as described below. More preferably, the compounds have an ED50 at least comparable to that of wild type bGH. Most preferably, the compounds have a higher affinity for growth hormone receptors than does the growth hormone native to the animal receiving the compound. For purification and characterization of a human growth hormone receptor, see Leung, et al., Nature, 330:537-43 (1987).

The preferred growth-inhibitory peptides are characterized by a modification of the surface topography of the third alpha helix. It will be seen from Figure 3 that in third alpha helix of "wild-type" bovine growth hormone, there is a surface cleft or depression beginning, at the Alanine-122, deepening at the Glycine-119, and ending with the Aspartate-115. All of the mutants prepared so far, both those which retain the wild-type growth-promoting activity and those which do not, are consistent with the theory that growth-promoting activity requires the presence of this cleft or depression and that, if it is "filled in" by substitution of amino acids with bulkier side chains, the mutein inhibits the growth of the subject.

Mutations which substantially destabilize the alpha-helix are undesirable since they may result in the loss of all growth-related activity. We have observed such loss in the case of several mutations which were expected to disrupt the alpha helix.

For a discussion of alpha helix formers and breakers, see Chou and Fasman, supra. Glu, Ala and Leu are the preferred alpha formers while Pro and Gly are characterized as strong helix breakers. Substitutions which introduce strong alpha helix breakers are less desirable, but may be tolerated in a particular case, such as the end of the helix. The secondary structures of our analogues have been predicted using the "Micro Genie" computer program, which uses the algorithm of Garnier, et al., J. Biol. Chem., 120:97-120 (1978).

With respect to amino acid 119, glycine is both the smallest amino acid residue and the one least favorable to alpha-helix formation. Thus, it is believed that any other amino acid may be substituted for it without destabilizing the alpha helix, while at the same time filling in the aforementioned cleft. It is possible that G119A will confer the "small animal" phenotype, particularly in conjunction with

other mutations, and such mutation is in that case within the scope of the present invention. However, alanine is less favored than other amino acids, because it is the next smallest amino acid and since alanine is the native amino acid at position 122. More preferably, the new amino acid is larger than alanine.

With regard to position 122, the alanine may be changed to any amino acid other than glycine (which has the disadvantages already mentioned), provided that the mutation does not destroy the alpha helix. Proline is not favored because mutations K114P, E118P and L121P are believed to destroy the alpha-helix. Tyrosine has a higher alpha-helical propensity than proline, but still lower than that of the wild-type alanine. However, since the alanine at position 122 is flanked on both sides by leucine, a strong alpha-helix former, it is possible that the substitution of tyrosine or even proline would be tolerated.

The modification of position 115 is suggested by our "cleft" theory. The aspartate at position 115 may be replaced by a bulkier amino acid which does not destroy the alpha helix. Preferably, the replacement amino acid has a size greater than that of glutamate. The amino acids histidine, methionine, isoleucine, leucine, lysine, arginine, phenylalanine, tyrosine and tryptaphan are substantially larger than glutamate. Of these, His, Met, Leu and Trp are more preferred because they combine the advantages of bulk with a reasonably strong alpha-helical propensity. Note, however, that the wild-type Glu is the strangest alpha-helix former of all of the amino acids.

The amino acids at positions 119, 122 and 115 may be altered singly or in combination. It is also possible to alter another amino acid in the alpha helix provided that the substitution does not destroy the alpha helix. Preferably, such alterations replace an amino acid with one of similar size and polarity. It may be advantageous to modify amino acids

flanking the primary mutation sites 119, 122 and/or 115 in order to increase the alpha-helical propensities of the sequence, particularly if the mutation at 119, 122 and/or 115 is one expected to destabilize the helix.

The present invention is not limited to any particular method of producing the desired bGH antagonists. Preferably, these antagonists are produced by first altering a gene encoding a BGH having the "native" third alpha helix by site-specific mutagenesis, and then cloning and expressing the altered gene in a suitable host. The gene may be of genomic origin, it may be cDNA prepared from bGH messenger RNA, it may be synthetic, or it may be a combination thereof.. For the amino acid sequence of bGH and for the cDNA sequence of the bGH gene, see Miller, et al., J. Biol. Chem., 255:7521-24 (1980). For the genomic sequence, see Woychick, et al., Nucleic Acids Res., 10:7197-7210 (1982).

The host may be any convenient organism, including a bacterial, yeast, or mammalian cell. The gene is operably linked to a promoter functional in the host. A constitutive promoter would activate gene expression in a general manner, i.e., in many tissue and at all times during development. A regulatable promoter may be activated in a tissue or cell specific manner, at precise time during development, or in response to changes in the environment. A constitutive promoter is usually employed when larger amounts of gene product (usually protein) is required or when the gene product is required in many cells of many tissues. A regulatable promoter is utilized when one gene product is required in a small number of cells of a particular tissue or at a given time during development.

The expression system may be engineered so that the antagonist is secreted into the culture medium, or the host cells may be grown to a high cell density and then lysed to release the compound.

One method suitable for the purification of bGH (G119R) and the like is described in Leung, et al., Endocrinology, 119:1489-1496 (1986). Essentially, this procedure involves purification by (a) ammonium sulfate precipitation, (b) fractionation on DEAE-cellulose (or any equivalent ion-exchange column), and (c) gel filtration (e.g., on a Sephadex G-25 and/or Sephacryl S-200 column). Other procedures applicable to purification of growth hormone-related compounds are set forth in Reichert, Jr., "Purification of Anterior Pituitary Hormones: Bovine, Rat and Rabbit," Meth. Enzymol., 37:360 et seq. (Academic Press, N.Y.:1975). Polyclonal or monoclonal antibodies which specifically recognize the protein of interest may also be used in the purification process.

The purified antagonist may then be combined with compatible, nontoxic pharmaceutical excipients and administered to an animal or human, e.g. to treat a condition characterized by an excessive growth rate. In the case of administration to animals, it may be preferable to incorporate the drug into the animal's feed, possibly in a prepared combination of drug and nutritional material ready for use by the farmer. An effective dosage and treatment protocol may be determined by conventional means, starting with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well. The trial dosages would be chosen after consideration of the clinical literature with respect to administration of growth hormones, and of somatostatin (a growth hormone release inhibitor).

In another embodiment, the gene is introduced into a host cell which is developed into genetically transformed cells of a transgenic animal. Linearized DNA bearing the growth hormone antagonist gene may be microinjected into a gamete, into the pronuclei of fertilized eggs, into the cytoplasm, into the nuclei of two-cell embryos, into individual cells of a

blastocyst, or into the blastocoel cavity. (Some of these targets may be reached by electroporation instead of microinjection.) Alternatively, a retrovirus bearing the gene may be constructed and used to infect preimplantation embryos or tissue culture cells (e.g., embryonic stem cells) which may be aggregated with such embryos. In either case, the genetically modified zygote, after a brief in vitro cultivation, is implanted into a foster mother and carried to term. For "gene therapy" post partum, see Cline, et al., Nature, 284:422-425 (1980); Williamson, Nature, 298:416-18 (1982). Again, the gene is operably linked to a promoter functional in the host, and the promoter may be constitutive or regulatable. Preferably, expression is regulated so abnormal embryonic or fetal development is avoided.

The invention is further illustrated, without limitation, by the following examples.

Example 1: Generation of Mutations Conferring the Reduced Growth Phenotype

MATERIALS AND METHODS

The plasmid, pBGH-10delta6, was derived from pBGH-10 and contains the complete coding region of bGH and intron A. Bovine growth hormone introns B, C and D are absent (Figure 1). This plasmid encodes "wild type" bGH, and its expression is controlled by a 1700 base pair segment of the mouse metallothionein I transcriptional regulatory sequence.

Plasmids pBGH-10delta6-$G^{119}R$ and pBGH-10delta6-$E^{117}L$, $G^{119}R$, $A^{122}D$ were derived from pBGH-10delta6 and were generated by segment-directed mutagenesis using complementary oligonucleotides to replace the DNA between the Tth111I site (found near the 3' end of Exon IV) and the Xma I site (located near the 5' end of Exon V). The other mutations described herein were generated similarly.

The complementary oligonucleotides used for pBGH10 delta 6-$G^{119}R$ were:
5'GTGTCTATGAGAAGCTGAAGGACCTGGAGGAAAGGATCCTGGCCTGATGCGGGAGCTGGA AGATGGCACCCC 3'; 73-MER) and (5'CCGGGGGGTGCCATCTTCCAGCTCCCGCAT CAGGGCCAGGATCCTTTCCTCCAGGTCCTTCAGCTTCTCATAGACA 3'; 76-MER).
The complementary oligonucleotides used for pBGH10delta6-$E^{117}L$, $G^{119}R$, $A^{122}D$ were:
(5'GTGTCTATGAGAAGCTGAAGGACCTGCTGGAAAGGATCCTGGACCTGATGCGGGAGCTG GAAGATGGCACCCC 3'; 73-mer) and 5' CCGGGGGGTGCCATCTTCCAGCTCCCGC ATCAGGTCCAGGATCCTTTCCAGCAGGTCCTTCAGCTTCTCATAGACA 3'; 76-mer).
These oligonucleotides hybridize as follows:

G119R

GT GTC TAT GAG AAG CTG AAG GAC CTG GAG GAA AGG ATC CTG GCC
ACA CAG ATA CTC TTC GAC TTC CTG GAC CTC CTT TCC TAG GAC CGG
Arg Val Tyr Glu Lys Leu Lys Asp Leu Glu Glu Arg Ile Leu Ala
CTG ATG CGG GAG CTG GAA GAT GGC ACC CC
GAC TAC GCC CTC GAC CTT CTA CCG TGG GGG GCC
Leu Met Arg Glu Leu

E117L, G119R, A122D

GT GTC TAT GAG AAG CTG AAG GAC CTG CTG GAA AGG ATC CTG GAC
ACA CAG ATA CTC TTC GAC TTC CTG GAC GTC CTT TCC TAG GAC CGG
Arg Val Tyr Glu Lys Leu Lys Asp Leu Leu Glu Arg 11e Leu Asp

CTG ATG CGG GAG CTG GAA GAT GGC ACC CC
GAC TAC GCC CTC GAC CTT CTA CCG TGG GGG GCC
Leu Met Arg Glu Leu

These oligonucleotides encode DNA changes which result in the substitutions of arginine for glycine at position 119 in pBGH-10delta6-G119R; and leucine for glutamate at position 117, arginine for glycine at position 119 and aspartate for alanine at position 122 in pBGH-10delta6-$E^{117}L$, $G^{119}R$, and $A^{122}D$. These amino acids were chosen because they have hydrophilic (arginine and aspartic acid) or hydrophobic (leucine) character [See Hopp and Woods, PNAS (USA), 78:3824-28

(1981)], positively (arginine) or negatively (aspartic acid) charged side chains [See Kaiser and Kezdy, Science acid) 223:249-55 (1984)], and high α-helical-forming potential [See Chou and Fasman, Ann. Rev. Biochem., 47:251-76 (1978)] furthering generation of an idealized amphiphilic α-helix [See Margalit, et al., J. Immunol., 138:2213-29 (1987); Brems, et al., Biochemistry 26:7774-78 (1987); Kaiser and Kezdy, supra; Chen, et al., PNAS (USA), 87:5061-65 (July 1990]. In addition, these oligonucleotide duplexes encode a silent base-pair change designed to create a unique BamHI restriction site which simplified screening procedures. The oligonucleotides were annealed and subcloned between the Tth111I and XmaI sites using standard procedures (Maniatis et al., Molecular Cloning (Cold Spring Harbor: (1982)). Mutant plasmid DNA's were identified by digestion with BamH1 restriction site which simplified screening procedures. The oligonucleotides were annealed and subcloned between the Tth111I and XmaI sites using standard procedures (Maniatis et al., Molecular Cloning (Cold Spring Harbor: 1982). Mutant plasmid DNA's were identified by digestion with BamH1.

The nucleotide sequence of the mutated bovine growth hormone target regions were determined by using the dideoxy chain-termination method with modified T7 DNA polymerase (Sequenase, United States Biochemical; Sanger et al., PNAS (USA), 74:5463-67 (1977)). Oligonucleotide primers for manual DNA sequencing were synthesized using the DuPont Coder #300 DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis, passive elution and concentration by ethanol precipitation. The oligonucleotide primers used for the direct sequencing analysis of the two mutants was the following: 18-mer (5'AAATTTGTCATAGGTCTG 3'). Briefly, 1-3μg of double-stranded plasmid DNA was denatured in the presence of 0.2N NaOH, and 10-20 pmoles of oligonucleotide primer was allowed to anneal (65°C, 2 min. followed by 30 min. slow cool) to the denatured template. A two-step polymerization was performed by using the modified T7 DNA polymerase which extends the

oligonucleotide-primed chain in the presence of dNTP's and deoxyadenosine 5'-[α-^{35}S] triotriphosphate (>1000 Ci/mmole, Amersham) followed by transfer of equal aliquots into each of four specific dideoxynucleotide mixes which randomly terminate chain elongation. Following addition of a formamide termination buffer to each reaction, the samples were incubated at 80°C for 2 min. and the DNA sequence was determined after size fractionation of the four sets of fragments by 10% polyacrylamide/8M urea electrophoresis and autoradiography.

Example 2: Expression in Mammalian Cells in Culture

Using the in vitro mutagenesis protocols described above, two mutant bGH genes were generated initially: one converts glycine^{119} to arginine ("G119R") and the second converts glutamate^{117} to leucine, glycine^{119} to arginine, and alanine^{122} to aspartate (E117L, G119R, A122D).

The plasmids encoding these mutations as well as wild type bGH DNA (pBGH10delta) were transiently introduced into cultured mouse L cells, which were subsequently analyzed for bGH expression. Following "western analysis", protein bands of approximately 22,000 daltons were observed for wild type bGH and bGH derived from the two mutant genes.

Mouse L cells were maintained in DMEM (Gibco) plus 10% calf serum and 25μg/ml gentamicin (Gibco). In this study, a modification of a previously described transfection procedure was employed (Lopata et al., Nucleic Acids Res., 12:5707-5717 (1984)). Briefly, 2μg of plasmid DNA was added to 1.0ml of DMEM containing 0.2mg DEAE-dextran. This solution was added to approximately 10^6 cells in a 35-mm tissue culture plate which had been washed previously with 2.0ml of DMEM. Following incubation of the cells for 1 hour at 37°C, the DNA-DEAE-dextran solution was removed and the cells "shocked" for 90 seconds with 2.0ml of 10% DMSO in Hepes buffered saline, at room temperature. Subsequently, the "shock" solution was

removed and cells washed with 2.0ml DMEM. Media containing 10% Nu-Serum (Collaborative Research) plus 50µg/ml gentamicin were changed daily. Culture fluids were stored at -20°C. For bGH binding assays, transfected cells were incubated in DMEM minus serum for 16 hours, after which the culture fluids were removed and frozen at -20°C.

Sodium dodecyl sulfate (SDS) PAGE analyses of secreted bGH have been described (Kopchick et al., DNA, 4:23-31 (1985); Kelder et al., Gene, 76:75-80 (1989). In this study, we used a polyclonal anti-bGH serum for "western" analysis.

Example 3: Growth Hormone Receptor Binding Studies

Culture fluids lacking serum were collected from cells transfected by pBGH-10delta6 (wild type bGH) and the mutant bGH genes. Following lyophilization of the culture media and bGH concentration determinations, competitive membrane binding studies were carried out as previously described (Smith & Talamants, J. Biol. Chem., 262:2213-19 (1987)). Liver membrane preparations from C57BL/6JxSJL hybrid mice of either sex (60-120 days old) were homogenized with a Brinkman Polytron in 4 volumes (w/v) of 0.3M sucrose, 10mM EDTA, 50mM Hepes, 0.1mM TPCK and 1mM PMSF at pH 8.0. The above step and all the following protocols were carried out at 4°C. The homogenate was centrifuged at 20,000xg for 30 min. and the supernatant was centrifuged at 100,000xg for 1 hour. The pellets were washed once with 10mM Hepes, pH 8.0 and recentrifuged. These pellets were resuspended in 10mM Hepes, pH 8.0, to a protein concentration of approximately 50mg/ml. The membranes were aliquoted, frozen on dry ice, and stored at -20°C. Membrane protein concentrations were determined by the Lowry protein assay (Lowry et al., J. Biol. Chem., 193:265-275 (1951)).

Competitive binding assays were performed using the following protocol. Microsomal membranes corresponding to three mgs. protein were incubated with 30,000 cpm/tube ^{125}I bGH (Cambridge Medical Diagnostics) and unlabeled bGH ranging from 0.3ml assay buffer (20mM Hepes, 10mM $CaCl_2$ 0.1% BSA, and 0.05% NaN_3 pH 8.0). All assays were performed in triplicate. After overnight incubation at room temperature, membrane bound hormone was separated from free hormone by the addition of 1 ml of ice cold assay buffer followed by centrifugation at 10,000xg for 20 min. Membrane pellets were then assayed for radioactivity. Specifically bound radioactivity was determined by subtraction from the value produced by incubation of membranes with 5μg unlabeled bGH (Smith and Talamants, 1987).

Effective doses which resulted in 50% displacement (ED50) of ^{125}I-bGH from the membrane preparations were determined. Mutant bGH encoded by pBGH-10delta6-$G^{119}R$ and pBGH10delta 6-$E^{117}L$, $G^{119}R$, $A^{122}D$ revealed an ED50 value similar to (the triple mutant) or higher than (G119R) wild type bGH.

Example 4: Transgenic Mouse Production

A series of transgenic mouse lines which contain wild type and mutant bGH genes were produced by standard microinjection techniques (McGrane et al., 1988). DNA extraction from mouse tails, dot blots, and serum determinations was as described (McGrane et al., 1988).

The genes contain the transcriptional regulatory sequences of the mouse metallothionein I promoter which has been shown to be active in liver tissue as well as other tissues of the transgenic mouse (Palmiter et al., Nature, 300:611-615 (1982)). Offspring generated by the microinjection procedure were assayed for bGH DNA by slot blot hybridization analysis. Mouse lines were generated which contain approximately one copy of the recombinant bGH DNA sequences

derived from pBGH-10delta6, (wild type), pBGH-10delta6-G^{119R}, and pBGH10delta6-E^{117L}, $G^{119}R$, $A^{122}D$. Serum from transgenic animals were assayed for bGH levels by the Western technique. All mice which expressed the wild type bGH transgene in serum also possessed a corresponding enhanced growth rate. Mice which expressed mutant bGH ($G^{119}R$ or $E^{117}L$, $G^{119}R$, $A^{122}D$) in serum were dramatically and significantly smaller. The growth rate for wild type bGH transgenic mice relative to control littermates was 1.5 while the ratio for the two bGH mutant mice to control littermates was ~0.6. In the case of the triple mutant, we have generated 10 founder mice that express the mutated bGH gene. The growth ratio between the transgenic and nontransgenic littermates ranged from 0.58 to 1.00. The degree of suppression of growth was directly related to the serum levels of the mutated bGH. Three founders have been bred that pass the trait to offspring; ≈50% of these offspring are positive for the gene and possess the corresponding small phenotype.

Since there was no change in binding affinity of bGH-M8 (the triple mutant) to mouse liver membranes when compared with wild-type bGH, it is possible that bGH contains distinct growth-promoting and receptor-binding domain(s). The three substitution mutations in bGH (bGH-M8) located with the third α-helix may have altered either one or more critical amino acids in or the local conformation of this potential growth-promoting domain, subsequently resulting in a reduction in bGH growth promoting activity. Amino acid sequence comparison among GHs and products of other members of the gene family revealed that Glu-117, Gly-119, and Ala-122 are relatively conserved in products of the GH gene family, (See Watahiki, et al., (1989) supra). Ala-122 is conserved only in GHs from nonprimate mammals as well as chickens, whereas Glu-117 is only conserved in GHs from mammals. Gly-119 is conserved among products of all members of the GH gene family, which includes prolactins and placental lactogens. Therefore, these amino acids may be important for the biological activities of GHs.

It has been demonstrated that many activities of GH are mediated through a family of peptides known as insulin-like growth factors (IGF), in particular IGF-1, which is believed to be produced primarily in the liver following GH binding to its receptor(s). (See Truesch, et al., Ann. Rev. Physiol., 47:443-67 (1985); Zapt, et al., Harm. Res., 24:121-130 (1986)). IGF-1 has been shown to decrease GH production in the pituitary by a classical negative feedback mechanism. (Leung, et al., Endocrinology, 119:1489-96 (1986)). One hypothesis to explain the growth suppression in pBGH10Δ6-M8 transgenic mice is that bGH-M8 is active as an in vivo antagonist to mouse GH (mGH), thereby suppressing mouse IGF-1 production. If this is true, then one would expect not only a reduction in serum mouse IGF-1 levels in bGH M8 transgenic mice but also an increase in mGH production in the pituitary. Preliminary results from immunoblot analysis of whole pituitary glands taken from bGH-M8 transgenic mice, bGH transgenic mice, and their nontransgenic littermates suggest that the pituitary glands in those growth-suppressed mice contain higher levels of mGH relative to their nontransgenic littermates. In contrast, mGH levels in bGH transgenic mice were largely depressed because mouse IGF-1 levels in serum of bGH transgenic mice increased up to twice as much as levels in serum of their nontransgenic littermates. Palmiter, et al., Science, 222:809-14 (1983). If our hypothesis were true, it would be the first example to our knowledge of an in vivo growth hormone antagonist and the first example of uncoupling of growth-promoting and receptor-binding activities of GHs.

Example 5: Screening of other Muteins of bGH

By similar procedures, muteins of bGH with alterations in the third alpha helix have been prepared and tested for secretion in L cells, and, in selected cases, their effect on the growth of transgenic mice, with the following results.

Mutants	L Cell Secretion	Mean Animal Transgenic/Nontransgenic Growth Ratio
wt bGH	yes	1.5
K112L,K114W(M1)	yes	1.5
K114P,E118P(M10)	no	1.0
L121P,E126G(M11)	no	1.0
K114P	no	
E118P	no	
L121P	no	
E126G	yes	*1.5
E117L(M4)	yes	*1.5
G119R(M6)	yes	0.7
A122D(M2)	yes	*0.8
E117L,G119R(M7)	yes	
E117L,A122D(M3)	yes	
E117L,G119R,A122D(M8)	yes	*0.7
V109D,Y110D,L116R	yes	
E111L,G119W	yes	
L121R,M124K	yes	
E111L,G119W,L121R,M124K	yes	
G119P	yes	*0.7
G119K	yes	*0.7
G119L	yes	*0.7
G119S	yes	
G119Q	yes	
G119E	yes	
G119W	yes	
G119M	yes	
A122T	yes	
A122P	yes	
A122S	yes	
D115V		
D115G		
D115A		

R125L	yes	
V109D, Y110D, L116R, L121R, M124K	no	
E111L, G119W, R125L	yes	*0.7
E111L, G119W, L121R, M124K	yes	
V109D, Y110D, L116K, R125L	no	
L123I	yes	

* Significantly different from nontransgenic littermates ($P<0.05$)

The mutants are described by giving the original amino acid, its position in the amino acid sequence of bGH, and the replacement amino acid, with the amino acids set forth according to the internationally accepted single letter code. George, et al., Protein Seq. Data Anal., 1:27-39 (1987).

The mutant K112L, K114W shows the effect of expanding the hydrophobic face of the helix. This mutant affects animal growth much as does wild type growth hormone.

The mutations K114P, E118P and L121P (and various combinations thereof) apparently destroy the alpha helix (Proline is a strong alpha helix breaker.) The growth-related biological activity is abolished. The mutation E126G is a special case; glycine is a helix breaker, but position 126 is at the end of the helix so the normal biological activity is retained. So, too is G119P; one strong helix breaker was substituted for an even stronger one.

The third alpha helix of wild type growth hormone diverges from a perfect amphiphilic alpha helix at three positions. First, at 117, Glu is a hydrophilic amino acid in the hydrophobic face. Second, at 119, Gly is a neutral amino acid in the hydrophilic face. Finally, at 122, Ala is a

hydrophobic amino acid in the hydrophilic face. The mutations E117L, G119R and A122D, separately or in combination, increase the amphiphilic character of the helix. G119R additionally increases the alpha-helical tendencies of the sequence.

Our initial hypothesis was that the growth-inhibitory activity of the mutants G119R and E117L/G119R/A122D was associated with the increased amphipathicity of the third alpha helix. We have since developed evidence that the amphipathicity of the third alpha helix is largely irrelevant to that activity.

(1) The single E117L, like wt bGH, produced large animals.

(2) Mutant G119P produced the small animal phenotype even though proline is as hydrophilic as glycine.

(3) Mutant G119L produced the small animal phenotype even though leucine is hydrophobic and therefore disrupts the hydrophilic face of the helix.

(54) Mutant E111L/G119W/R125L produced the small animal phenotype even though all three mutations disrupt the hydrophilic face of the helix.

Thus, in one embodiment, the present invention relates to mutations of the third alpha helix which result in growth-inhibitory activity yet reduce or leave unchanged the amphiphilic character of the helix.

To summarize, residues 119 and 122 are important to the growth response, but alterations in these residues apparently did not affect receptor binding. Destruction of the helix by insertion of a helix-breaker at residues 114, 118 and

121 abolished both growth-affecting activity and, in cultured mouse cells, secretory activity. Residues 112, 114, 117 and 121 do not appear to be important in the growth response, at least so long as the helix is not destroyed.

Additional growth hormone antagonists may be identified by systematically varying the codon corresponding to G119 in bGH, so as to express the 18 other mutants having a single amino acid change at this position. This is readily accomplished by synthesizing oligonucleotides differing from those set forth in Example 1 at codon 119 so as to encode the desired alternative amino acid. By similar means, variations of the codons corresponding to amino acids 115 and 122 of bGH, or still other amino acids, are investigated.

The following table may be helpful in identifying candidate mutants:

AA	Volume (angstrom^3)	Alpha Helicity	Notes
Gly(G)	60.1	0.53	E126G(big), L121P/E126G(null)
Ala(A)	88.6	1.45	
Ser(S)	89.0	0.79	
Cys(C)	108.5	0.77	
Asp(D)	111.1	0.98	A122D(sm)
Thr(T)	116.1	0.82	
Asn(N)	117.7	0.73	
Pro(P)	122.7	0.59	G119P(sm), K114P/E118P(null), L121P/E126G(null)
Glu(E)	138.4	1.53	
Val(V)	140.0	1.14	
Gln(G)	143.9	1.17	
His(H)	153.2	1.24	
Met(M)	162.9	1.20	
Ile(I)	166.7	1.00	
Leu(L)	166.7	1.34	G119L(sm), E117L(big), E117L/G119R/A122D(sm), E111L/G119W/E125L (sm)
Lys(K)	168.6	1.07	G119K(sm)
Arg(R)	173.4	0.79	G119R(sm)
Phe(F)	189.9	1.12	
Tyr(Y)	193.6	0.61	
Trp(W)	227.8	1.14	E111L/G119W/R125L (sm)

See Schulz and Schirmer, Principles of Protein Structure, Tables 6-1 (1979), for alpha-helicity and see Creighton, Proteins: Structures and Molecular Properties, Table 1-1 (1983) for volume of amino acids. Table 1-2 of Creighton also gives references for the detailed geometries of the amino acids. In notes, "sm" = small animal, "big" = big animal, "null" = no effect on growth.

Claims

1. A peptide or protein of at least 11 amino acids, comprising an alpha helix which is substantially homologous with but not identical to the third alpha helix of bovine growth hormone (bGH), wherein at least one of the differences from the third alpha helix of bGH is at a residue corresponding to residues 115, 119 or 122 of bGH.

2. A protein according to claim 1, said protein being substantially homologous with bovine growth hormone.

3. The protein of claim 2 wherein the difference at position 115, 119 and/or 122 is the substitution of an amino acid which is bulkier than the corresponding amino acid of wild-type growth hormone.

4. The protein of claim 3 wherein the amino acid corresponding to amino acid 119 is selected from the group consisting of all naturally occurring amino acids except glycine and alanine.

5. The protein of claim 3 wherein the amino acid corresponding to amino acid 122 is selected from the group consisting of all naturally occurring amino acids except glycine and alanine.

6. The protein of claim 3 wherein the amino acid corresponding to position 115 is selected from the group consisting of histidine, methionine, isoleucine, leucine, lysine, arginine, phenylalanine, tryosine and trytophan.

7. A protein selected from the group consisting of
bGH (G119R),
bGH (G119R, E117L, A122D),
bGH (A122D),
bGH (G119P),
bGH (G119K),
bGH (G119L), and
fragments thereof having growth-inhibitory activity in a mammal.

8. A recombinant DNA molecule comprising a gene including a sequence encoding the peptide or protein of claim 1.

9. The molecule of claim 8, further comprising a promoter operably linked to said gene.

10. The molecule of claim 9 where said promoter is not derived from the promoter of a growth hormone gene.

11. A host cell transformed by the recombinant DNA molecule of claim 9.

12. The transformed cell of claim 11, where said host cell is one which does express a chromosomal growth hormone gene.

13. A nonhuman transgenic animal comprising a plurality of transformed cells according to claim 11.

14. The animal of claim 13, said animal exhibiting a reduced growth rate as compared to control animals.

15. A method of producing a growth inhibitory peptide or protein which comprises providing cells bearing a gene including a sequence encoding a peptide or protein

according to claim 1 with conditions conducive to the expression of said gene, whereby said protein is produced in usable or recoverable form.

16. The method of claim 14 wherein said protein is selected from the group consisting of

bGH (G119R),
bGH (G119R, E117L, A122D),
bGH (A122D),
bGH (G119P),
bGH (G119K),
bGH (G119L), and
fragments thereof having growth-inhibitory activity in a mammal.

17. A pharmaceutical composition comprising the peptide or protein of claim 1 and a pharmaceutically acceptable carrier.

18. A method of treating a condition of a human or animal subject characterized by an excessive growth rate which comprises administering to the subject a growth inhibitory amount of the composition of claim 16.

19. A method of treating a condition of a human or animal subject characterized by an excessive growth rate which comprises introducing into the subject recombinant DNA molecules according to claim 9, whereby the genetically modified subject is capable of expressing said protein at a growth inhibitory level.

20. The peptide or protein of claim 1, said peptide or protein having an ED50 which is less than about 10 times the ED50 of wild type bGH in an assay of the ability of the peptide or protein to displace radiolabeled wild type bGH from a mouse liver membrane receptor.

21. The peptide or protein of claim 20, said peptide or protein further characterized by growth-inhibitory activity.

22. The peptide or protein of claim 20 wherein the affinity of the peptide or protein for such receptors is greater than that of wild type bGH.

23. A method of identifying a vertebrate growth-affecting peptide or protein analogue of a vertebrate growth hormone which comprises cloning a gene encoding said analogue into a mouse L cell, said gene being operably linked to a promoter functional in such cells, expressing said analogue from said gene, and determining whether the analogue is secreted.

24. A protein which is substantially homologous with a vertebrate growth hormone but has growth-inhibitory activity.

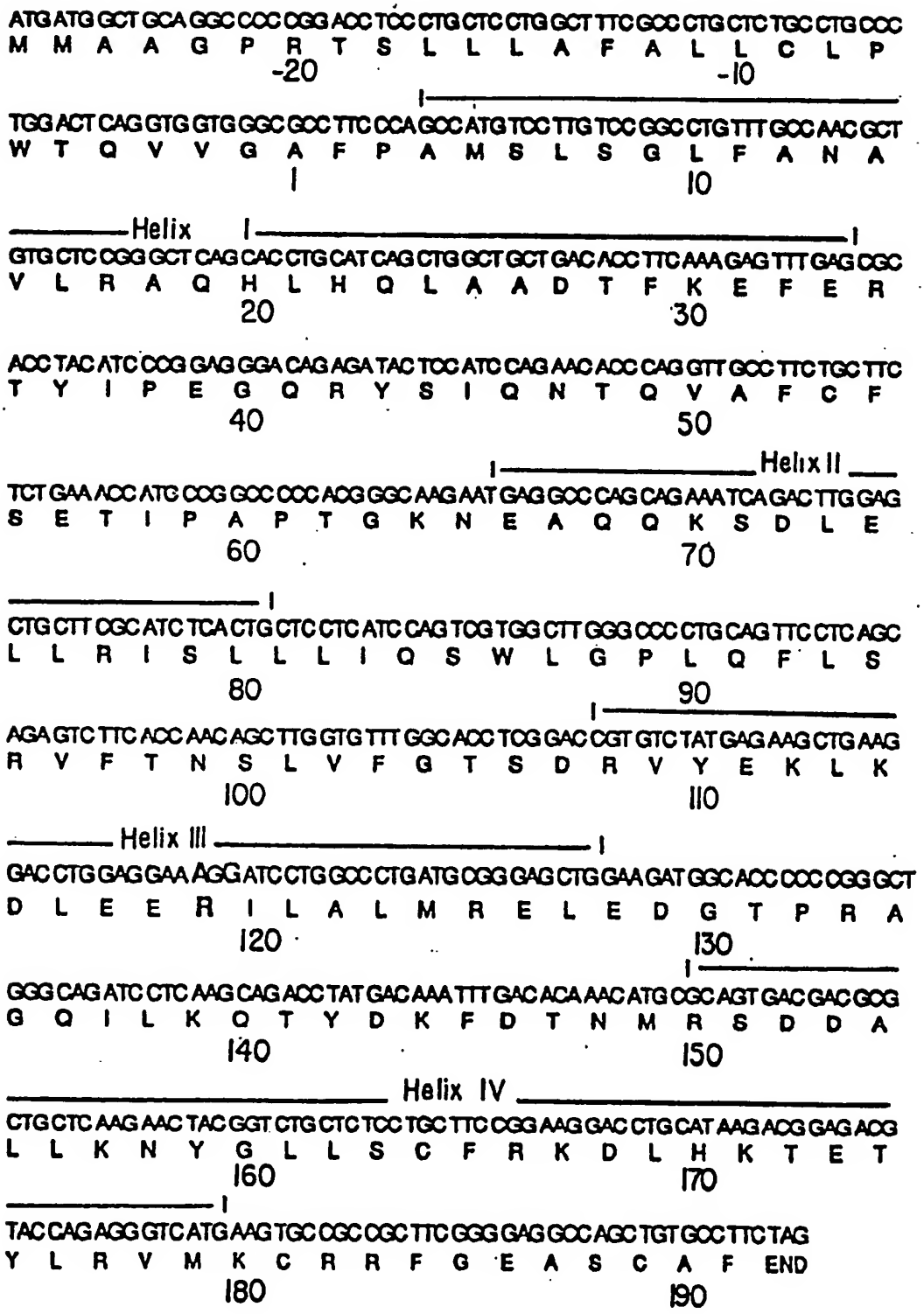

ATG ATG GCT GCA GGC CCC CGG ACC TCC CTG CTC CTG GCT TTC GCC CTG CTC TGC CTG CCC
M M A A G P R T S L L L A F A L L C L P
-20
-10
TGG ACT CAG GTG GTG GGC GCC TTC CCA GCC ATG TCC TTG TCC GGC CTG TTT GCC AAC GCT
W T Q V V G A F P A M S L S G L F A N A
1
10
Helix I
GTG CTC CGG GCT CAG CAC CTG CAT CAG CTG GCT GCT GAC ACC TTC AAA GAG TTT GAG CGC
V L R A Q H L H Q L A A D T F K E F E R
20
30
ACC TAC ATC CCG GAG GGA CAG AGA TAC TCC ATC CAG AAC ACC CAG GTT GCC TTC TGC TTC
T Y I P E G Q R Y S I Q N T Q V A F C F
40
50
Helix II
TCT GAA ACC ATC CCG GCC CCC ACG GGC AAG AAT GAG GCC CAG CAG AAA TCA GAC TTG GAG
S E T I P A P T G K N E A Q Q K S D L E
60
70
CTG CTT CGC ATC TCA CTG CTC CTC ATC CAG TCG TGG CTT GGG CCC CTG CAG TTC CTC AGC
L L R I S L L L I Q S W L G P L Q F L S
80
90
AGA GTC TTC ACC AAC AGC TTG GTG TTT GGC ACC TCG GAC CGT GTC TAT GAG AAG CTG AAG
R V F T N S L V F G T S D R V Y E K L K
100
110
Helix III
GAC CTG GAG GAA AGG ATC CTG GCC CTG ATG CGG GAG CTG GAA GAT GGC ACC CCC CGG GCT
D L E E R I L A L M R E L E D G T P R A
120
130
GGG CAG ATC CTC AAG CAG ACC TAT GAC AAA TTT GAC ACA AAC ATG CGC AGT GAC GAC GCG
G Q I L K Q T Y D K F D T N M R S D D A
140
150
Helix IV
CTG CTC AAG AAC TAC GGT CTG CTC TCC TGC TTC CGG AAG GAC CTG CAT AAG ACG GAG ACG
L L K N Y G L L S C F R K D L H K T E T
160
170
TAC CAG AGG GTC ATG AAG TGC CGC CGC TTC GGG GAG GCC AGC TGT GCC TTC TAG
Y L R V M K C R R F G E A S C A F END
180
190

FIG. 1.

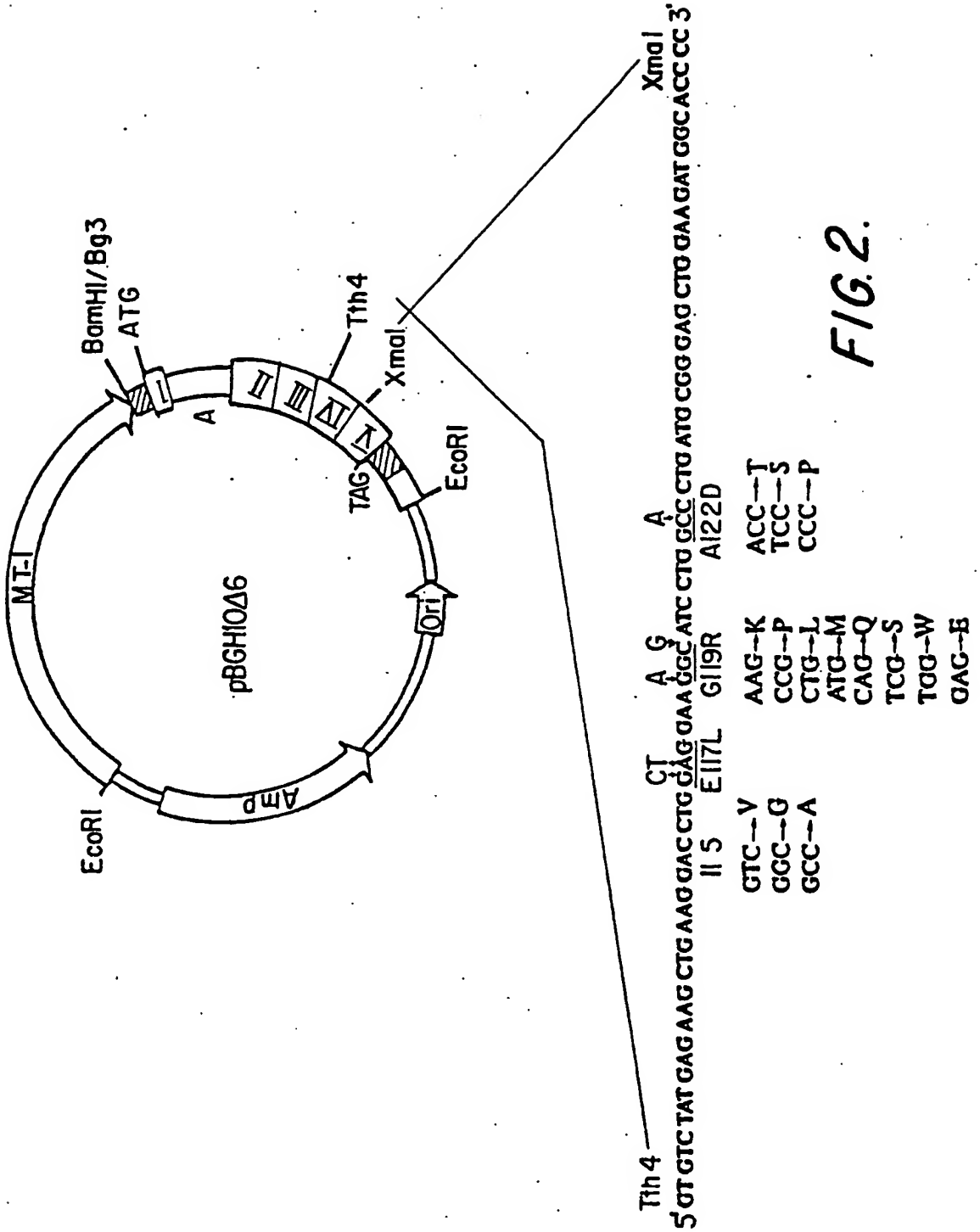
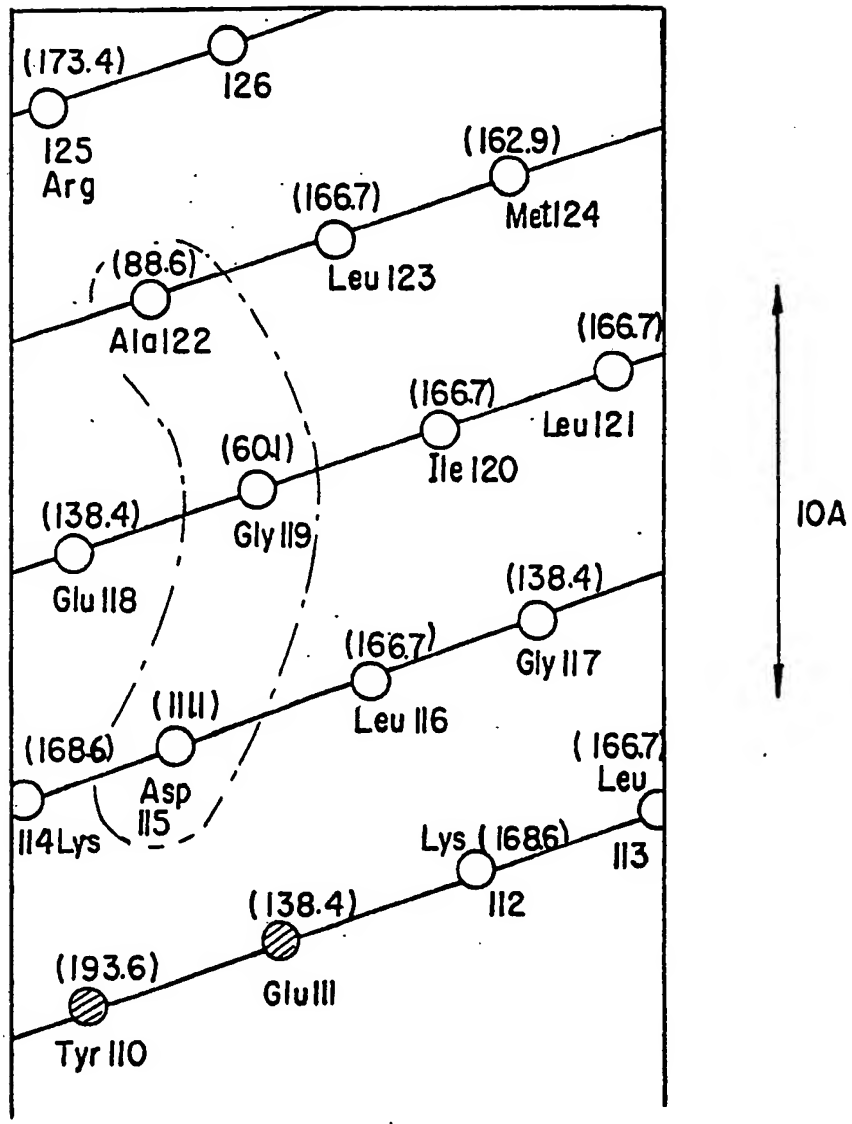

pBGH10Δ6
MT-I
BamHI/Bg3
ATG
EcoRI
Amp
Ori
TAG
Tth4
Xmal
EcoRI
A
I
II
III
IV
V
Xmal
Tth4
5'GT GTC TAT GAG AAG CTG AAG GAC CTG GAG GAA GGC ATC CTG GCC CTG ATG CGG GAG CTG GAA GAT GGC ACC CC 3'
CT
A G
A
II 5 E117L G119R A122D
GTC—V AAG—K ACC—T
GGC—G CCG—P TCC—S
GCC—A CTG—L CCC—P
ATG—M
CAG—Q
TCG—S
TGG—W
GAG—E

FIG. 2.

FIG. 3.


(173.4)
126
125
Arg
(162.9)
(166.7)
Met124
Leu 123
(88.6)
Ala122
(166.7)
(166.7)
Leu 121
Ile 120
(60.1)
(138.4)
Gly 119
Glu 118
(138.4)
(166.7)
Gly 117
Leu 116
(111.1)
(168.6)
(166.7)
Leu
Asp
115
114 Lys
Lys (168.6)
113
(138.4)
112
(193.6)
Glu 111
Tyr 110
10A

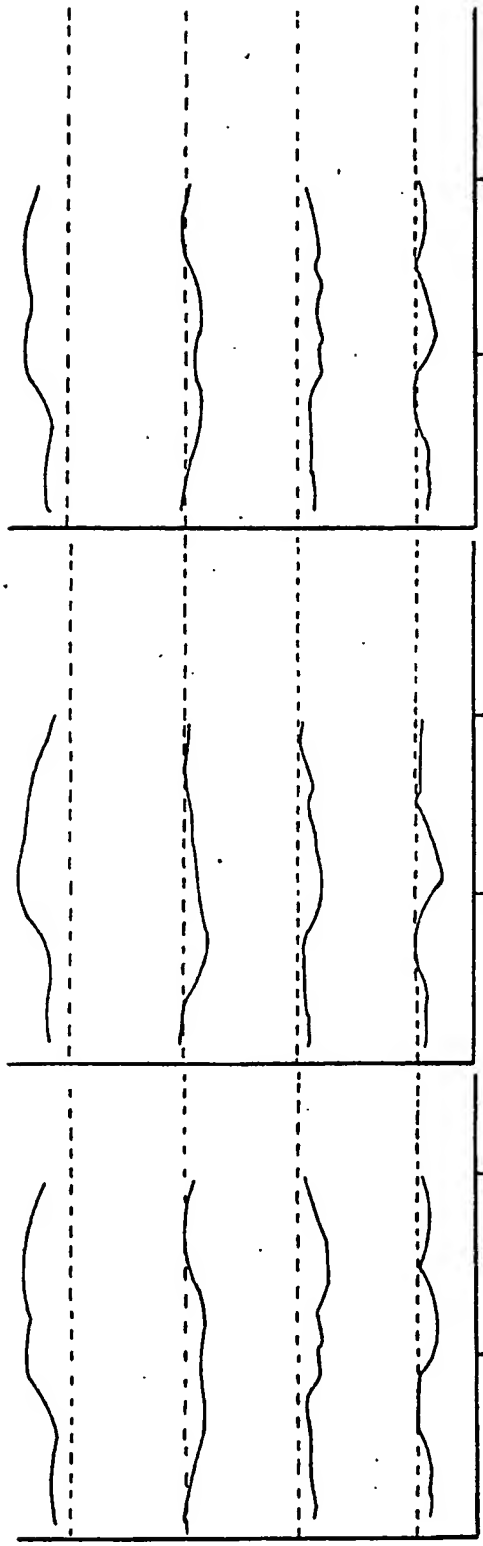


FIG. 4a.

FIG. 4b.

FIG. 4c.

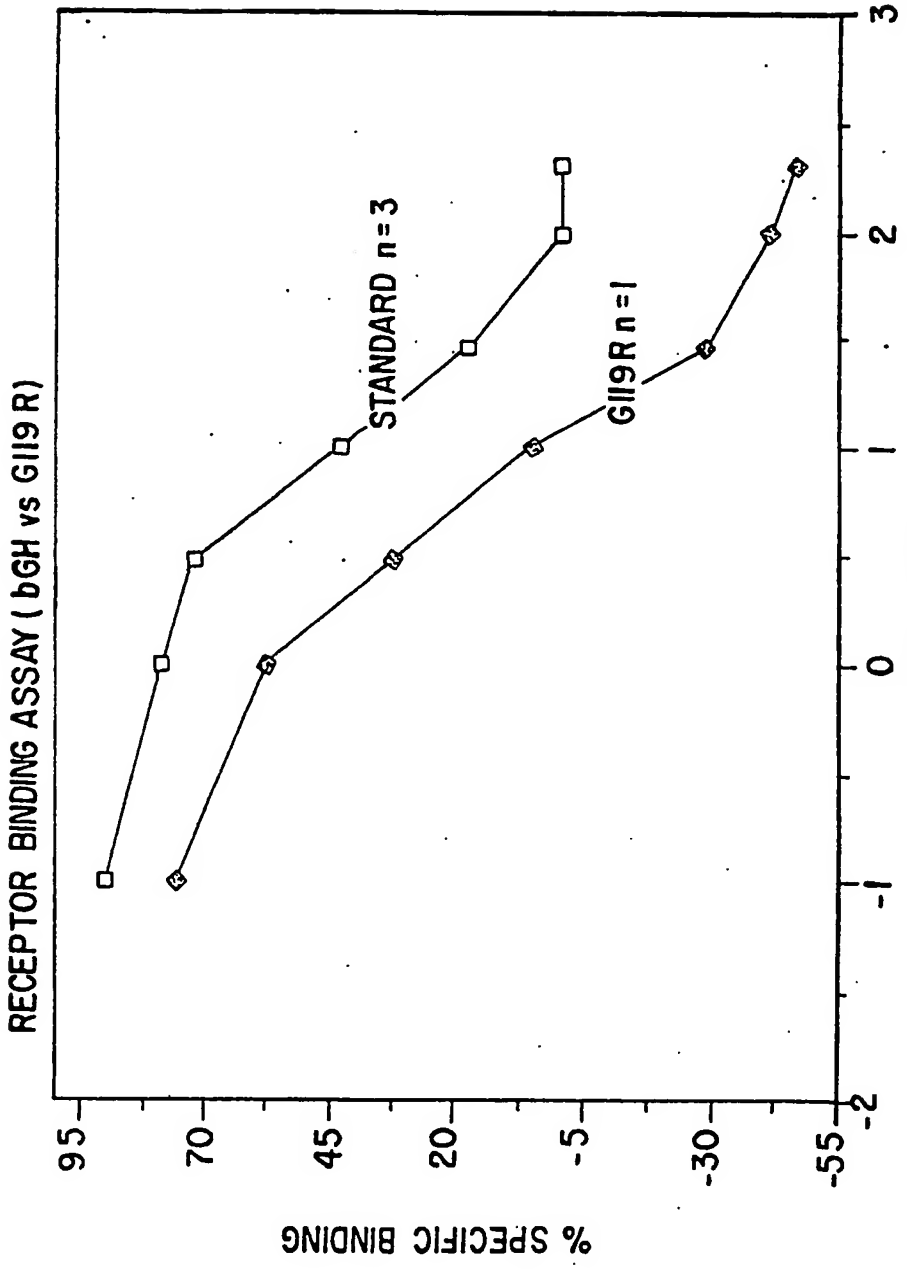

RECEPTOR BINDING ASSAY (bGH vs GII9 R)
% SPECIFIC BINDING
95
70
45
20
-5
-30
-55
3
2
1
0
-1
-2
LOG (bGH)
STANDARD n=3
GII9R n=1

FIG.5.

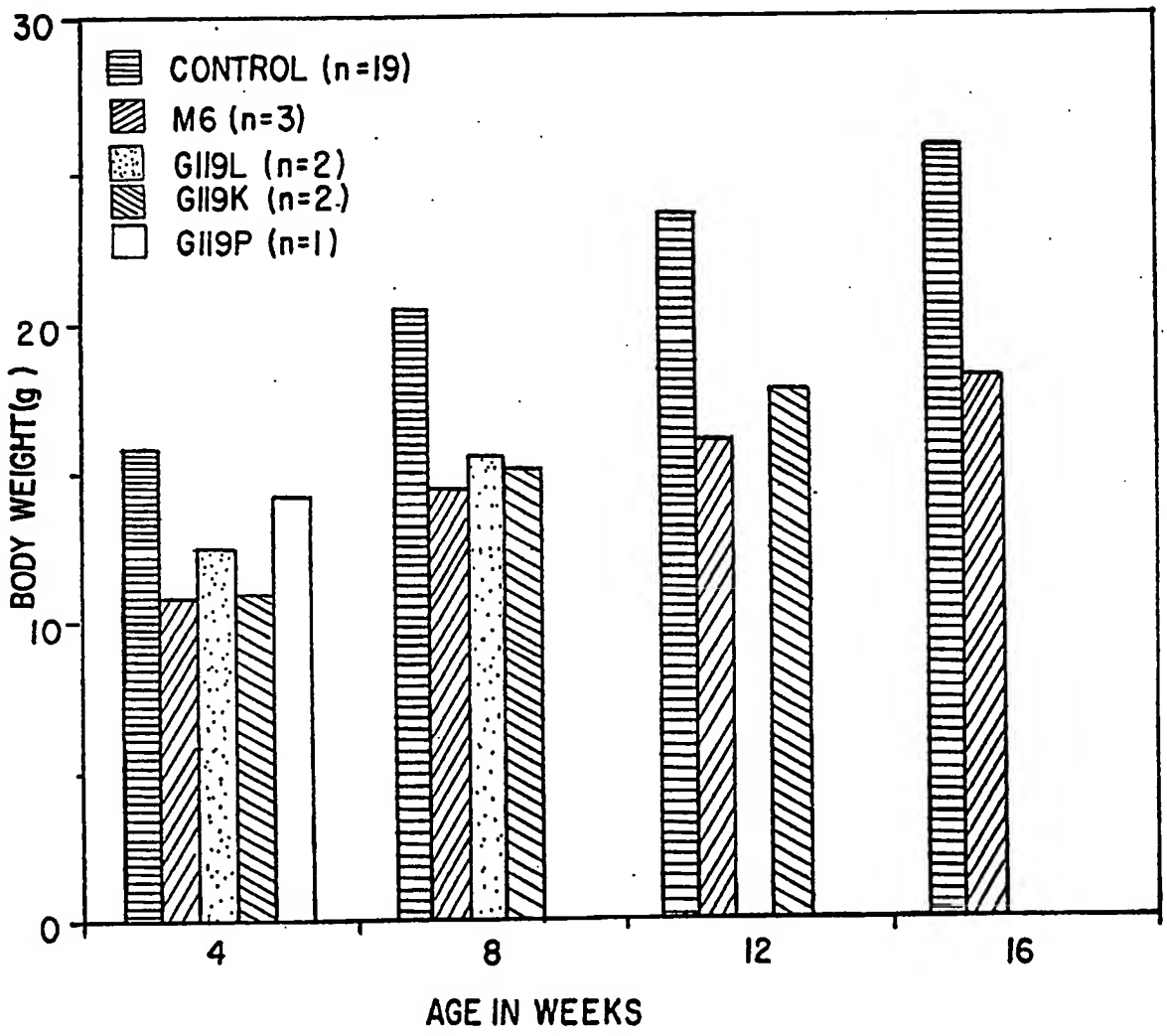

ON GOING STUDY AT POSITION 119 OF bGH FEMALE
CONTROL (n=19)
M6 (n=3)
G119L (n=2)
G119K (n=2)
G119P (n=1)
BODY WEIGHT(g)
30
20
10
0
4
8
12
16
AGE IN WEEKS

FIG.6.

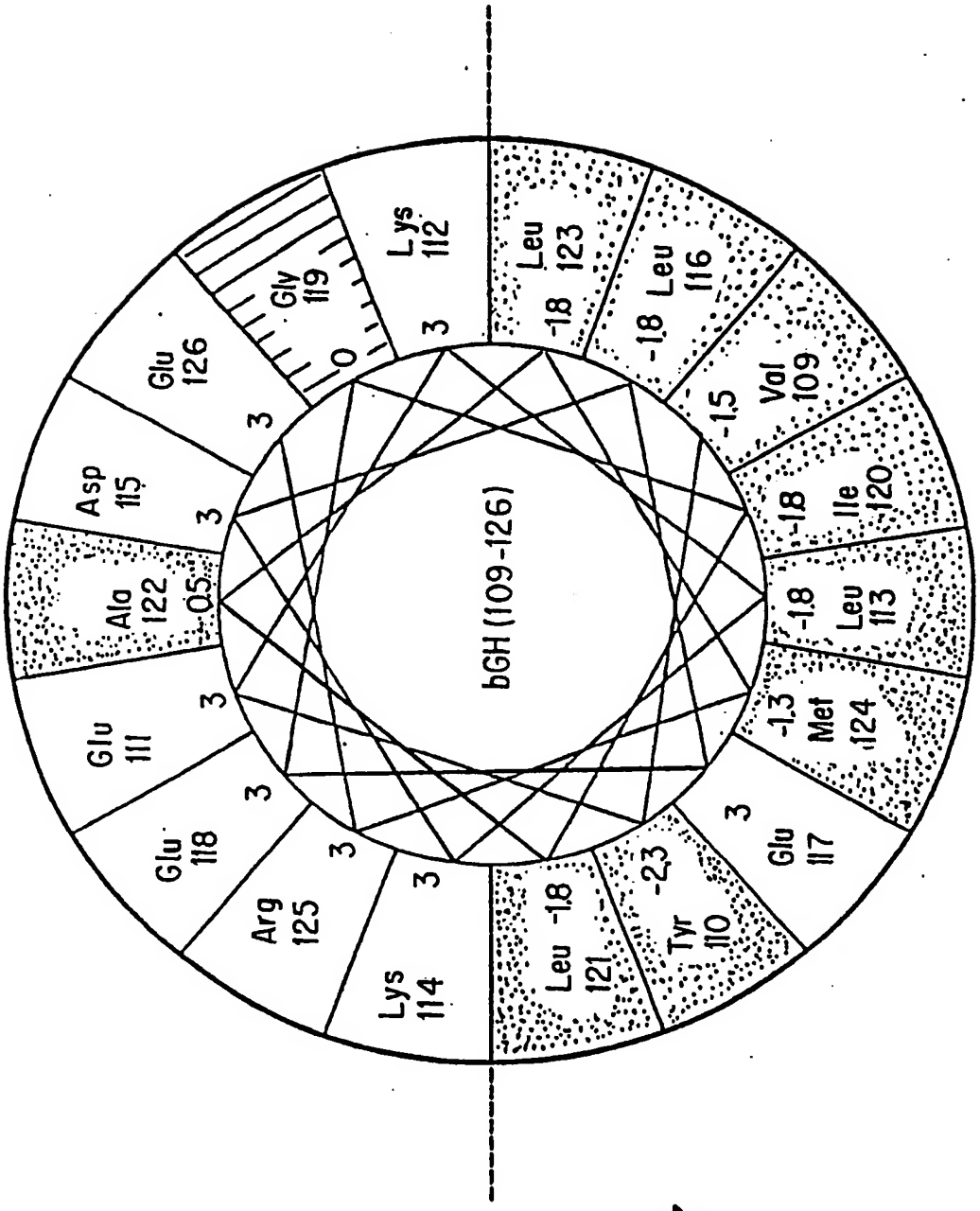

bGH (109-126)
Lys 112 3
Leu 123 -1.8
Leu 116 -1.8
Val 109 -1.5
Ile 120 -1.8
Leu 113 -1.8
Met 124 -1.3
Glu 117 3
Tyr 110 -2.3
Leu 121 -1.8
Lys 114 3
Arg 125 3
Glu 118 3
Glu 111 3
Ala 122 -0.5
Asp 115 3
Glu 126 3
Gly 119 0

FIG. 7.

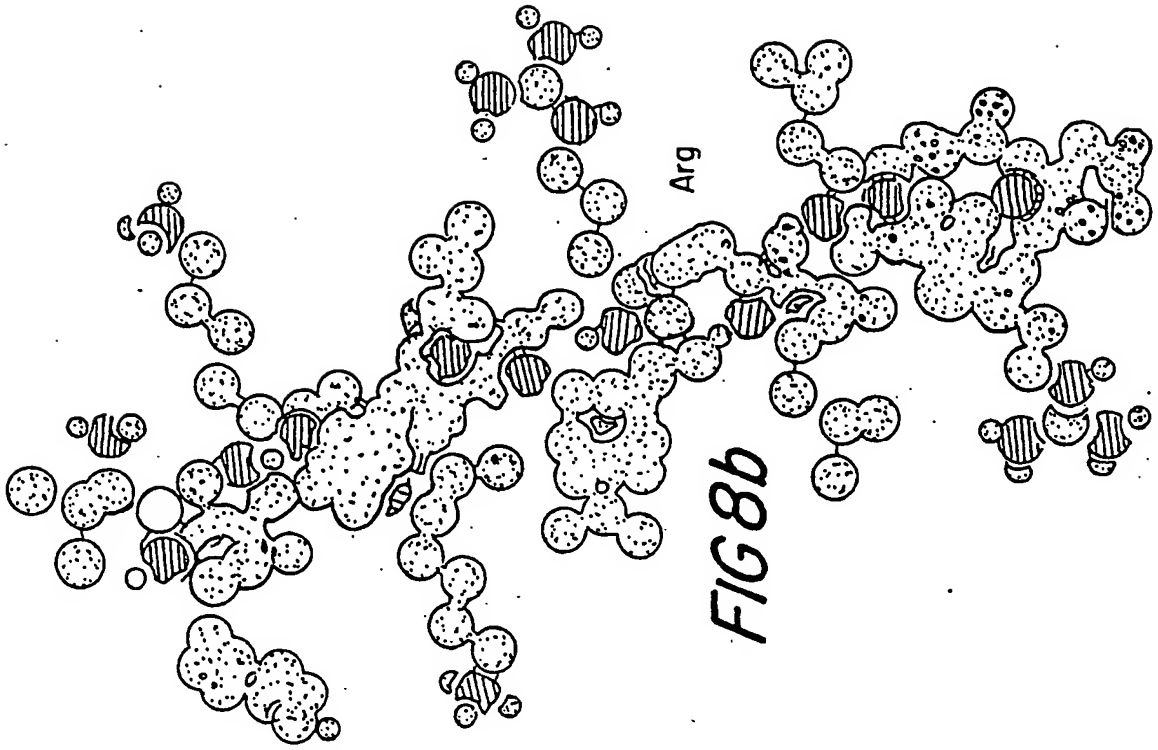


FIG8b

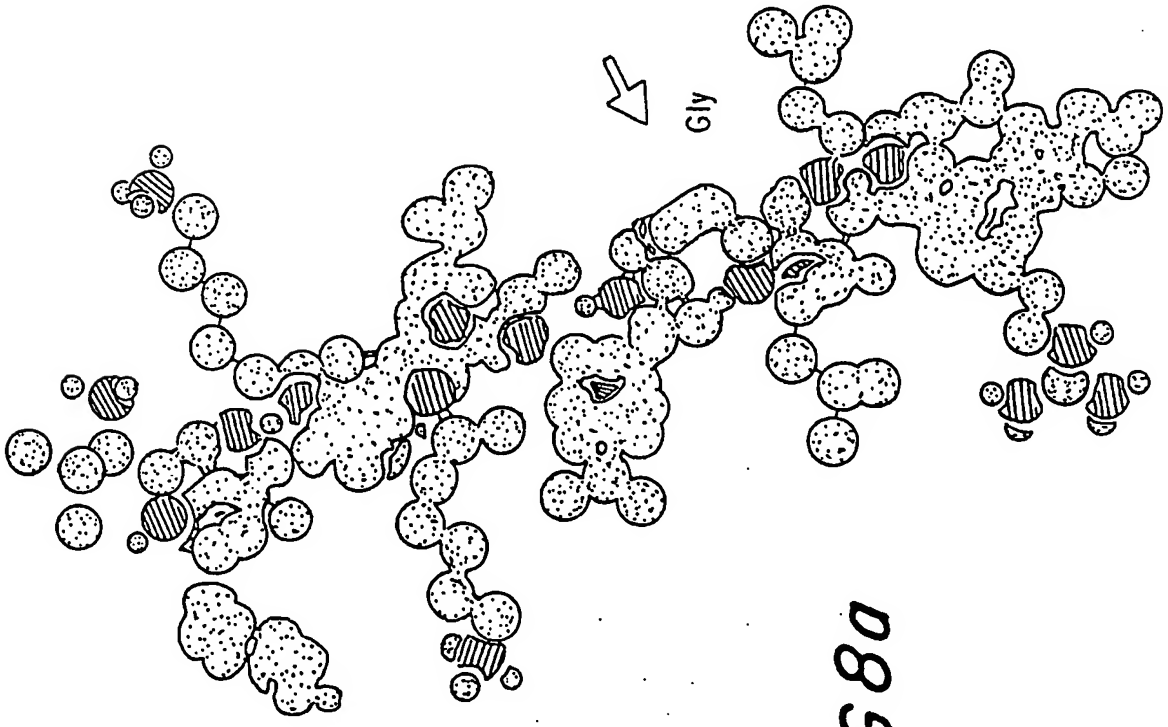


FIG8a

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/05874

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 5/00, 15/00; A61K 37/00, 48/00; C07K 13/00

U.S. Cl: 435/69.1, 240.2; 536/27; 514/2, 44; 530/399; 800/2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/69.1, 240.2, 172.3, 317.1; 536/27; 514/2, 44; 800/2, DIG 1; 530/350, 399

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Database: DIALOG (Files 55, 311, 312, 154), USPTO Automated Patent System (File USPAT, 1975-1991). See attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X, P Y	Proceedings of the National Academy of Sciences, Volume 87, issued July 1990, **CHEN ET AL**, "Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice, pages 5061-5065, see the entire document.	1-5,7-16,20,24 6, 17-19
Y	Proceedings of the National Academy of Sciences, Volume 85, issued May 1988, **BREMS ET AL.**, "Stabilization ofan associated folding intermediate of bovine growth hormone by site-directed mutagenesis", pages 3367-3371, see the entire document.	1-24
Y	Biochemistry, Volume 26, No. 24 issued 1987, **BREMS ET AL.**, "Helical Formation in Isolated Fragments of Bovine Growth Hormone", pages 7774-7778, see the entire document.	1-24
Y	Biochemical and Biophysical Research Communications, Volume 139, No. 2, issued 16 September 1986, **TOU ET AL.**, "Amphiphilic growth hormone releasing factor (GRF) analog: peptide design and biological activity invivo", pages 763-770, see the entire document.	1-24

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

04 February 1991

Date of Mailing of this International Search Report

04 MAR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Jasemine C. Chambers

Jasemine C. Chambers

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	"Protein Engineering", Published 1987, **ALAN R. LISS, INC.** (New York, USA), pages 193-199, see the entire document.	1-24
A	Science, Volume 243, issued 10 March 1989, **CUNNINGHAM ET AL.**, "Receptor and Antibody Epitopes in Human Growth Hormone Identified by Homolog-Scanning Mutagenesis", pages 1330-1336, see the entire document.	1-24
Y	Nature, Volume 315, issued 20 June 1985, **HAMMER ET AL.**, "Production of transgenic rabbits, sheep and pigs by microinjection", pages 680-683, see the entire document.	11-16, 19, 23

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(See attachment)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, Volume 300, issued 16 December 1982, **PALMITER ET AL.**, "Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes", pages 611-615, see the entire document.	11-16, 19, 23
Y	Science, Volume 222, issued 18 November 1983, **PALMITER ET AL.**, "Metallothionein-Human GH Fusion Genes Stimulate Growth of Mice", pages 809-814, see the entire document.	11-16, 19, 23

Attachment to PCT/ISA/210, Part II

II FIELDS SEARCHED SEARCH TERMS:

growth hormone,

mutein

mutant

mutagenesis

transgenic

dwarf

express

bovine

inventor's name

Attachment to PCT/ISA/210
Part VI. Observations where unity of invention is lacking

I. Claims 1-7, 17, 18, 20-22 and 24, drawn to a protein, a pharmaceutical composition and a method of using the same, classified in Classes 530 and 514, subclasses 399 and 2, respectively, for example.

II. Claims 8-16, 19 and 23, drawn to a DNA molecule, a transformed cell, a transgenic animal and methods of using the DNA molecule or transformed cell, classified in Classes 536, 435 and 800, subclasses 27 69.1 and 2, respectively, for example.

Attachment to PCT Telephone Memorandum
Reasons for Holding Lack of Unity of Invention

The invention defined by Group II is not limited in use to the preparation of the invention of Group I and can be used for the preparation of nucleic acid probes, for example. The preparation of the invention of Group I does not require the invention of Group II since the protein can be made by chemical sequence synthesis.

United States Patent [19]

McCulloch et al.

[11] Patent Number: 5,071,560

[45] Date of Patent: Dec. 10, 1991

[54] **PROCESS FOR PURIFYING PHENYLALANINE**

[75] Inventors: Beth McCulloch, Barrington; Walter H. Goodman, Villa Park, both of Ill.

[73] Assignee: UOP, Des Plaines, Ill.

[21] Appl. No.: 631,175

[22] Filed: Dec. 19, 1990

Related U.S. Application Data

[63] Continuation of Ser. No. 380,921, Jul. 17, 1989, abandoned, which is a continuation-in-part of Ser. No. 260,105, Oct. 20, 1988, abandoned.

[51] Int. Cl.[5] B01D 15/08

[52] U.S. Cl. 210/635; 210/656; 435/108; 562/443

[58] Field of Search 562/443; 435/108; 210/635, 656

[56] **References Cited**

U.S. PATENT DOCUMENTS

2,985,589	5/1961	Broughton et al.	210/34
3,040,777	6/1962	Carson et al.	137/625.15
3,422,848	1/1969	Liebman et al.	137/625.15
3,663,467	5/1972	Albright	260/2.5 B
3,706,812	12/1972	De Rosset et al.	260/674 SA
3,787,317	1/1974	Jaworek	210/31 C
4,537,763	8/1985	Miyake	426/658
4,584,400	4/1986	Otani et al.	562/443
4,604,483	8/1986	Kitsukawa et al.	562/443
4,616,078	10/1986	Di Marchi	530/305
4,642,397	2/1987	Zinnen et al.	568/934

OTHER PUBLICATIONS

Summary Bulletin—Amberlite Polymeric Adsorbents, Rohm & Haas (11 pages), 1978.

Preliminary Technical Notes—Amberlite ® XAD-7 , Rohm & Haas (13 pages) 1978.

Primary Examiner—Ernest G. Therkorn

Attorney, Agent, or Firm—Thomas K. McBride; John F. Spears, Jr.; Jack H. Hall

[57] **ABSTRACT**

A process for the liquid phase adsorptive separation of phenylalanine from a fermentation broth containing phenylalanine salts, carbohydrates, amino acids and organic acids. The feed is contacted, at a pH of 4.5–6.5, with a hydrophobic polar, porous synthetic adsorbent, such as Amberlite XAD-7, whose functional groups have a dipole moment of 1.6–2.0, to selectively adsorb the phenylalanine onto said adsorbent to the substantial exclusion of the other feed components and recovering phenylalanine by desorbing with water, an alcohol, a ketone or an ester.

12 Claims, No Drawings

PROCESS FOR PURIFYING PHENYLALANINE

This is a continuation of copending application Ser. No. 07/380,921 filed on July 17, 1989, which, in turn, is a continuation-in-part of U.S. Ser. No. 260,105, filed Oct. 20, 1988, both now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The field of art to which this invention pertains is the solid bed adsorptive separation of phenylalanine. More specifically, the invention relates to a process for separating and recovering L-phenylalanine (hereinafter "phenylalanine") from an aqueous solution such as a fermentation broth employing a synthetic polar adsorbent to selectively adsorb phenylalanine.

2. Description of the Prior Art

Phenylalanine is an essential amino acid and is used in the synthetic production of pharmaceuticals and more recently extensively in the production of "Aspartame", a non-nutritive sweetener sold under the trade name "Nutrasweet". There are several routes to the production of phenylalanine: the fermentation of sugar; the enzymatic conversion of cinnamic acid, hydantoin or other sources, e.g., phenylacetaldehyde. All of these routes produce phenylalanine, together with other reaction products, such as a lactic acid, acetic acid, phenyllactic acid, cinnamic acid and hydrocinnamic acid, salts, such as KCl, sugars, other amino acids and organic acids.

In U.S. Pat. No. 4,584,400, a process for separating L-phenylalanine from a fermentation broth by a chromatographic process with non-polar adsorbents, e.g., XAD-2 and XAD-4 is disclosed, where the predominant contaminant is L-tyrosine. However, enormous volumes of water, the desorbent, are required to desorb phenylalanine.

Phenylalanine has also been separated from cinnamic acid, as disclosed in U.S. Pat. No. 4,604,483, utilizing XAD-2, XAD-4, XAD-7 and XAD-8 in the presence of at least 0.1N solution of a salt, e.g., ammonium chloride. In this process, the selectivity of the adsorbent for the two components is reversed due to the greater salting-out effect of ammonium chloride on the cinnamic acid than on the phenylalanine. Thus, phenylalanine is eluted first with substantially no cinnamic acid. Applicant's invention does not rely on the salting-out effect on the selectivity.

U.S. Pat. No. 3,787,317 discloses the use of at least two different chromatographic materials, e.g., dextran-based molecular sieves, usually crosslinked, to separate mixtures which are stated to include phenylalanine.

A technical bulletin (undated) promulgated by Rohm and Haas Company discusses the use of Amberlite XAD-7 in several separations, viz. fatty acids from water or toluene; phenol or m-chlorophenol from water or toluene; proteins from aqueous fluids of biological origin. One of these general suggestions for separations is more specifically disclosed in U.S. Pat. No. 4,616,078, wherein proinsulin-like substances may be separated from impure mixtures obtained by recombinant DNA methodology by adsorption on Amberlite XAD-7 or XAD-8 and elution with acetone or acrylonitrile under specified conditions.

None of the references disclose an effective and economic chromatographic process for separating phenylalanine from a fermentation broth.

The invention herein can be practiced in fixed or moving adsorbent bed systems, but the preferred system for this separation is a countercurrent simulated moving bed system, such as described in Broughton U.S. Pat. No. 2,985,589, incorporated herein by reference. Cyclic advancement of the input and output streams can be accomplished by a manifolding system, which are also known, e.g., by rotary disc valves shown in U.S. Pat. Nos. 3,040,777 and 3,422,848. Equipment utilizing these principals are familiar, in sizes ranging from pilot plant scale (DeRosset U.S. Pat. No. 3,706,812 to commercial scale and flow rates from a few cc's per hour to many thousands of gallons per hour.

The functions and properties of adsorbents and desorbents in a chromatographic separation of liquid components are well known, but for reference thereto, Zinnen et al. U.S. Pat. No. 4,642,397 is incorporated herein.

SUMMARY OF THE INVENTION

The present invention is a process for separating phenylalanine from a fermentation feed comprising phenylalanine, salts, carbohydrates, amino acids and organic acids. The process comprises contacting, at adsorption conditions, the feed mixture, while maintaining the pH of the feed mixture from 4.5 to 6.5, with a hydrophobic, polar, porous synthetic adsorbent, and selectively adsorbing phenylalanine onto said adsorbent to the substantial exclusion of the other feed components and desorbing, under desorption conditions, the phenylalanine with desorbent which comprises water, an alcohol, a ketone or an ester or mixtures thereof.

DETAILED DESCRIPTION OF THE INVENTION

The adsorbent to be used in the process of this invention will comprise a group of specific non-ionic hydrophobic synthetic crosslinked aliphatic polymers. It is not an ion exchange resin since it contains no ionically functional groups, but rather, derives its adsorptive properties from the combination of macroreticular porosity, pore size distribution, high surface area and the aliphatic nature of its structure. A preferred adsorbent in this group is a macroporous, crosslinked acrylic ester copolymer which has intermediate polarity and more specifically, where the functional groups have a dipole moment of 1.8. The nature of adsorbents having intermediate polarity is described in Rohm & Haas' *Summary Bulletin—Amberlite Polymeric Adsorbents*, page 1 and Table 1, page 3. The dipole moment of representative adsorbents is given in Table 2, page 5. However, it is believed that the dipole moment of the group of adsorbents defined above may vary, e.g., from about 1.6 to about 2.0. Typical crosslinking agents (a comonomer) may include divinylbenzene, but polyfunctional aliphatic monomers e.g., methacrylic acid, acrylic acid or derivatives thereof, etc., are preferred. A more preferred adsorbent is a self-crosslinked acrylic ester homopolymer from monomers having polyfunctional groups, i.e., at least three methacrylate groups, capable of self-crosslinking, e.g., trimethylolpropane trimethacrylate and pentaerythritol tetramethacrylate, etc. XAD-8 is a copolymer of methyl acrylate and trimethylolpropane trimethacrylate. Examples of self-crosslinking polyfunctional monomers include the aforementioned trimethylolpropane trimethacrylate. The preferred copolymers and homopolymers and the method of making them are disclosed in U.S. Pat. No. 3,663,467, incorporated herein by reference.

As previously stated, there are several synthetic routes to the production of phenylalanine, but all result in a mixture of products from which phenylalanine must be separated. A suitable feed is the fermentation product of a carbohydrate source, such as sugar, which has been treated by ultrafiltration to remove certain of the impurities such as residual cells, cell debris, etc. The feed may contain, among other components, salts, such as potassium chloride and ammonium phosphate, $(NH4)_2HPO_4$, sugars, including glucose, and maltose, organic acids, e.g., lactic, phenyllactic acids and hydrocinnamic, amino acids, such as phenyl-alanine, alanine and lysine. The concentration of salts is usually less than 0.1N although in some feeds may be 0.5N or greater. Nevertheless, greater concentrations can be separated in this process since the salts are not adsorbed and eluted at the void volume and recovered in the raffinate.

It is an important aspect of the process to maintain the pH in the range where the phenylalanine is present as a zwitterion and is hydrophobic. At this pH, most of the other components present in the feed will be hydrophilic and will elute at the void volume. The preferred pH will be in the range of 4.5 to 6.5 with a pH of 6 being most preferred.

Illustrative of the adsorbent which may be used in our invention is Amberlite XAD-7, a self-crosslinked homopolymer made from the monomer, trimethylolpropane trimethacrylate, obtained from Rohm & Haas Co. as hard insoluble beads of 20–50 mesh, having a surface area of 450 m^2/g, average pore diameter of 80 Å and a porosity of 0.5 to 0.55 ml/g. XAD-8 has a porosity, or pore volume, of 0.52 ml/g, surface area of 140 m^2/g, average pore diameter of 235 Å and can be obtained in mesh sizes of 25–50. Since Amberlite XAD-7 and XAD-8 have an aliphatic structure, they are more hydrophilic than other prior art nonionic hydrophobic polymers derived from aromatic monomers, such as the crosslinked polystyrene polymers exemplified by Amberlite XAD-4. Both XAD-7 and XAD-8 have dipole moments of the functional groups of 1.8. Conversely, the dipole moments of the functional groups of XAD-2 and XAD-4, all as reported by Rohm and Haas, the manufacturer, in Summary Bulletin-Amberlite Polymeric Adsorbents (undated), are 0.3. The adsorbents are not limited to those mentioned above, but any other polar, highly porous, crosslinked aliphatic synthetic adsorbent having the same properties can be used.

In the process of the present invention, the fermentation feed mixture containing phenylalanine, salts, carbohydrates, other amino acids and organic acids are brought into contact with a polar hydrophobic crosslinked aliphatic synthetic polymer to thereby adsorb the phenylalanine on said polymeric resin adsorbent and desorbing the phenylalanine adsorbed onto the resin by contacting the adsorbent with a desorbent comprising water, an alcohol, a ketone or an ester, or mixtures thereof. The separation process may be either batch or continuous and preferably in a fixed or moving adsorbent bed system, with the most preferred system being a countercurrent simulated moving bed system, such as described in the aforementioned Broughton U.S. Pat. No. 2,985,589. As previously mentioned, in a typical countercurrent simulated moving bed system, cyclic advancement of the input and output streams are accomplished by a manifolding system, e.g., by rotary disc valves showing U.S. Pat. Nos. 3,040,777 and 3,422,848, in sizes ranging from pilot plant scale to commercial scale.

The instant process represents an improvement over prior art processes in which a non-ionic, non-polar synthetic adsorbent, based on crosslinked polystyrene, was used, as disclosed in the aforementioned U.S. Pat. No. 4,584,400. In Example 2 of said patent, fourteen liters or bed volumes of 1% aqueous ethanol solution was required for elution of the L-phenylalanine. Such a long retention volume is a prohibitive factor in commercialization of such a process; however, utilizing the adsorbent disclosed herein, a greater than 10-fold reduction in retention volume can be achieved, rendering the process commercially viable and quite advantageous. Moreover, with the use of stronger desorbents, such as alcohols, ketones and esters, the retention volume can be reduced even further. For example, alcohols, such as methanol, ethanol, propanol, etc. are suitable. Ketones, such as acetone, methyl ethylketone are also usable. Also, esters, such as ethylacetate, propyl acetate, butyl acetate, etc. are expected to reduce the retention volume by the greatest degree.

A dynamic testing apparatus is employed to test various adsorbents with a particular feed mixture and desorbent material to measure the adsorption characteristics of retention capacity and exchange rate. The apparatus consists of a helical adsorbent chamber of approximately 100 cc volume having inlet and outlet portions at opposite ends of the chamber. The chamber is contained within a temperature control means and, in addition, pressure control equipment is used to operate the chamber at a constant predetermined pressure. Quantitative and qualitative analytical equipment such as refractometers, polarimeters and chromatographs can be attached to the outlet line of the chamber and used to detect quantitatively or determine qualitatively one or more components in the effluent stream leaving the adsorbent chamber. A pulse test, performed using this apparatus and the following general procedure, is used to determine data, e.g., selectivity, for various adsorbent systems. The adsorbent is placed in a chamber and filled to equilibrium with a particular desorbent material by passing the desorbent material through the adsorbent chamber. At a convenient time, a pulse of feed containing known concentrations of a tracer and of a particular extract component or of a raffinate component or both, all diluted in desorbent material is injected for a duration of several minutes. Desorbent material flow is resumed, and the tracer and the extract component or the raffinate component (or both) are eluted as in a liquid-solid chromatographic operation. The effluent can be analyzed on-stream or alternatively, effluent samples can be collected periodically and later analyzed separately by analytical equipment and traces of the envelopes or corresponding component peaks developed.

From information derived from the test, adsorbent performance can be rated in terms of void volume, retention volume for an extract or a raffinate component, the rate of desorption of an extract or a raffinate component from the adsorbent, the resolution between the components and selectivity for one component with respect to the other. The retention volume of an extract or a raffinate component may be characterized by the distance between the center of the peak envelope of the extract or raffinate component and the center of the peak envelope of the tracer component (void volume) or some other known reference point. It is expressed in terms of the volume of desorbent material pumped during this time interval represented by the distance between the peak envelopes. The rate of exchange or

desorption rate of an extract component with the desorbent material can generally be characterized by the width of the peak envelopes at half intensity. The narrower the peak width, the faster the desorption rate. Selectivity, β, is determined by the ratio of the net retention volumes of the more strongly adsorbed component to each of the other components.

Resolution is a measure of the degree of separation of a two-component system, and can assist in quantifying the effectiveness of a particular combination of adsorbent, desorbent, conditions, etc. for a particular separation. Resolution for purposes of this application is defined as the distance between the two peak centers divided by the average width of the peaks at ½ the peak height as determined by the pulse tests described hereinafter. The equation for calculating resolution is thus:

$$R = \frac{L_2 - L_1}{1/2(W_1 + W_2)}$$

where L_1 and L_2 are the distance, in ml, respectively, from a reference point, e.g., zero to the centers of the peaks and W_1 and W_2 are the widths of the peaks at ½ the height of the peaks.

Although both liquid and vapor phase operations can be used in many adsorptive separation processes, liquid-phase operation is preferred for this process because of the lower temperature requirements and because of the higher yields of extract product than can be obtained with liquid-phase operation over those obtained with vapor-phase operation. Adsorption conditions will include a temperature range of from about 20° C. to about 200° C. with about 50° C. to about 90° C. being more preferred and a pressure range of from about atmospheric to about 500 psig (3450 kPa gauge) being preferred to ensure liquid phase. Desorption conditions will include the same range of temperatures and pressures as used for adsorption.

The examples shown below are intended to further illustrate the process of this invention without unduly limiting the scope and spirit of said process. The examples present test results for various adsorbent and desorbent materials when using the above dynamic testing apparatus.

EXAMPLE I

A pulse test, as described above, was run at 58° C. on a series of feeds comprising 5 ml each of a 2% aqueous solution of each of the following pure components: phenylalanine, lactic acid, phenyllactic acid, glucose and KCl. The adsorbent was Amberlite XAD-7 (Rohm & Haas) having particle sizes of 20–50 mesh. After each feed pulse was introduced, the desorbent water at a pH of 5 was introduced into the column at a flow rate of 2 ml/min. In this example, a total volume of 165 ml water was used to desorb the entire amount of phenylalanine adsorbed onto the adsorbent. The results are shown in the following Table 1.

TABLE 1

Component Name	Gross Retention Volume (ml)	Net Retention Volume (ml)	Peak Width At Half-Height (ml)	Separation Factor (Beta)	Resolution Factor (0.5 Height)
KCl	83.4	0	16.5	1.48	1.54
Glucose	86.1	2.7	21.9	1.44	1.31
Lactic	84.1	0.7	19.6	1.47	1.43
PHE lactic	107.8	24.4	26.6	1.15	0.51

TABLE 1-continued

Component Name	Gross Retention Volume (ml)	Net Retention Volume (ml)	Peak Width At Half-Height (ml)	Separation Factor (Beta)	Resolution Factor (0.5 Height)
PHE	123.6	40.2	35.5	Refer.	Refer.

EXAMPLE II

Another pulse test was run at 58° C. using an actual fermentation product having the following analysis:

TABLE 2

Composition	Wt. %
Phenylalanine	2.8
Other Amino Acids (Hydrolyzed)	0.30
NH_3	0.27
Sugars	0.8
Salts	1.17
Lactic Acid	1.0
Phenyllactic Acid	0.2
Total Solids (%) (Calculated)	6.5
Total Solids (%) (Measured)	7.5

Feed consisted of 5 ml of the above composition at a pH of 6.1. The desorbent was water at a pH of 7.5 and the flow rate of 2 ml/min. The results are shown in the following Table 3. The amount of water required to completely desorb the phenylalanine was less than 165 ml.

TABLE 3

Component Name	Gross Retention Volume (ml)	Net Retention Volume (ml)	Peak Width At Half-Height (ml)	Separation Factor (Beta)	Resolution Factor (0.5 Height)
Salt 1	83.2	0	16.8	1.45	1.38
Salt 2	88.1	4.9	23.1	1.37	1.08
Lactic	90.9	7.7	13.5	1.33	1.173
Unknown 1	96.6	13.4	14.5	1.25	0.93
Unknown 2	122.6	39.4	23	0.99	0.05
PHE	121	37.8	38	Refer.	Refer.

EXAMPLE III

Fermentation broth was used in a further pulse test, this time at a pH of 5, using a feed mixture having the composition in the following Table 4. Complete desorption of the phenylalanine required about 175 ml.

TABLE 4

Composition	Wt. %
Phenylalanine	2.8
Other amino acids (Hydrolyzed)	0.3
Sugars	1.1
Salts	1.66
Lactic Acid	0.1
Phenyllactic Acid	0.1
Total Solids (%) (Calculated)	6.06
Total Solids (%) (Measured-Micro Wave)	8.1

The results are shown in the following Table 5.

TABLE 5

Component Name	Gross Retention Volume (ml)	Net Retention Volume (ml)	Peak Width At Half-Height (ml)	Separation Factor (Beta)	Resolution Factor (0.5 Height)
Salt 1	81.4	0	15.4	1.51	1.39
Salt 2	85.8	4.4	18.7	1.43	1.18
Lactic	88.9	7.5	15.9	1.38	1.13
PHE lactic	110.5	29.1	21	1.11	0.39
Unknown	123.4	42.0	23.4	1.00	0.01
PHE	123.1	41.7	44.3	Refer.	Refer.

What is claimed is:

1. A method for separating phenylalanine from a fermentation feed comprising phenylalanine, salts, carbohydrates, amino acids and organic acids, comprising contacting said feed, while maintaining said feed at a pH of about 4.5 to about 6.5, with a hydrophobic, porous synthetic adsorbent having functional groups whose dipole moment is from 1.6 to 2.0, a surface area of 140 to 450 m^2/g, an average pore diameter of 80 to 235 Angstroms and a porosity of 0.5 to 0.55 ml/g, comprising a macroporous acrylic ester polymer, adsorbing said phenylalanine onto said adsorbent, removing said other feed components from contact with said adsorbent as raffinate, and desorbing said phenylalanine at desorption conditions with a desorbent comprising water, an alcohol, a ketone or an ester.

2. The method of claim 1 wherein said adsorbent is a crosslinked, macroporous acrylic ester copolymer.

3. The method of claim 2 wherein the crosslinking comonomer is trimethylolpropane trimethacrylate.

4. The method of claim 1 wherein said adsorbent is a crosslinked acrylic ester homopolymer.

5. The method of claim 4 wherein the monomer from which said homopolymer is derived is trimethylolpropane trimethacrylate.

6. The method of claim 1 wherein said desorption conditions comprise temperatures within the range of 40° to 90° C.

7. The method of claim 1 wherein said pH is about 6.

8. The method of claim 1 wherein the concentration of salts in said feed is less than about 0.1N.

9. The method of claim 1 wherein the concentration of salts in said feed is less than about 0.5N.

10. The method of claim 1 wherein the dipole moment of the functional groups of said adsorbent is 1.8.

11. The method of claim 10 wherein the desorbent is water.

12. The method of claim 1 wherein said adsorbent has a surface area of about 450 m^2/g, an average pore diameter of about 80 Angstroms, and a porosity of about 0.5 to 0.55 ml/g.

* * * * *